Universidade do Minho
Escola de Ciências

Rita Isabel Costa Cunha

The mechanism mediating nuclear translocation of the apoptosis inducing factor (AIF)

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Tese de Mestrado em Genética Molecular

Trabalho efectuado sob a orientação de
Professora Doutora Manuela Côrte-Real
Doutora Susana Chaves

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DECLARAÇÃO

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The mechanism mediating nuclear translocation of the apoptosis inducing factor (AIF)

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1. **É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE, APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.**

Universidade do Minho, 31 de Outubro de 2012

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Rita Isabel Costa Cunha

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The mechanism mediating nuclear translocation of the apoptosis inducing factor (AIF)

Abstract

Since the discovery that yeast cells can undergo programmed cell death in response to several different stimuli, *Saccharomyces cerevisiae* has gained prominence in the cell death field. Exposure of yeast cells to certain stimuli like acetic acid or hydrogen peroxide or even heterologous expression of pro-apoptotic proteins can trigger cell death by apoptosis via a mitochondrial pathway and exhibiting the typical hallmarks of apoptosis, such as chromatin condensation, ROS (reactive oxygen species) accumulation, DNA fragmentation, externalization of phosphatidylserine, mitochondrial dysfunction with release of cytochrome *c* and of apoptosis inducing factor (Aif1p), among other pro-apoptotic proteins.

Aif1p is a flavoprotein that is involved both in cell survival and cell death. In healthy cells, Aif1p is confined to the mitochondria, where it plays a role in bioenergetic and redox metabolism due to its redox activity and its FAD and NADH domains. However, when cells are exposed to an apoptotic stimulus, Aif1p is released from mitochondria to the cytosol and then translocated to the nucleus, where it will induce DNA fragmentation and chromatin condensation. Although the mechanism underlying this translocation is still poorly understood, it is known that transport into the nucleus through the nuclear pore complexes is an energy-dependent process for the majority of macromolecules, and normally mediated by the major class of transport receptors, the karyopherins or Kaps. In this work, we aimed elucidate the mechanism regulating yeast Aif1p import into the nucleus and to discover which Kap is responsible for the nuclear import of Aif1p.

Apoptosis was triggered in cells expressing Aif1p tagged with GFP (by exposure to acetic acid or hydrogen peroxide, chronological ageing, or heterologous expression of Bax *c*-myc protein) and Aif1p localization was assessed by fluorescence microscopy and cellular fractionation/western blot assays. However, we were not able to observe the release of Aif1p to the cytosol or its nuclear import under our experimental conditions. We also attempted to map the NLS domain of Aif1p by deletion of several domains with homology to previously mapped domains in human AIF. Although we has not yet mapped the Aif1p NLS domain, we discovered that deletion of the putative Hsp70p-binding domain led to aberrant localization of Aif1p, suggesting that Hsp70p is important for Aif1p folding and stability. These results indicate that Hsp70p may also be involved in the regulation of Aif1p localization in yeast.

Mecanismo mediador da translocação nuclear do fator de indução de apoptose (AIF)

Resumo

Células de *Saccharomyces cerevisiae* são capazes de desencadear um processo de morte celular programada em resposta a vários estímulos. Esta descoberta levou à utilização extensiva deste organismo eucarionte unicelular como modelo no estudo de processos de morte celular. Sabe-se que a exposição de células de levedura a certos estímulos, como o ácido acético ou peróxido de hidrogénio, ou mesmo a expressão heteróloga de proteínas pró-apoptóticas de mamífero, pode desencadear um tipo morte celular dependente de uma via mitocondrial e exibindo características típicas da morte apoptótica, tais como a condensação da cromatina, acumulação de espécies reativas de oxigénio (ROS), fragmentação do DNA, externalização de fosfatidilserina e disfunção mitocondrial associada à libertação de citocromo *c* e do fator indutor de apoptose (Aif1p).

Aif1p é uma flavoproteína que está envolvida tanto na sobrevivência como na morte celular. Em células saudáveis, esta proteína está confinada à mitocôndria, onde desempenha um importante papel no metabolismo bioenergético e estado redox. Este facto é devido à sua atividade redox e aos seus domínios FAD e NADH. No entanto, em células expostas a um estímulo apoptótico, ocorre libertação de Aif1p da mitocôndria para o citosol e subsequentemente a sua translocação para o núcleo, onde induz fragmentação do DNA e condensação da cromatina. Embora o mecanismo subjacente a esta translocação esteja ainda pouco conhecido e estudado, sabe-se que para a maioria das macromoléculas o transporte para o núcleo ocorre através dos complexos de poros nucleares e é um processo dependente de energia, normalmente mediado por uma classe de recetores de transporte, as Kaps (do inglês “karyopherins”, também conhecidas como importinas ou transportinas). Este trabalho teve como objectivo elucidar a regulação do mecanismo de transporte de Aif1p para o núcleo e identificar que/ais Kap(s) são responsáveis pelo importe do Aif1p para o núcleo na levedura.

A morte celular apoptótica foi induzida em células que expressam uma fusão da proteína Aif1 com GFP (por exposição a ácido acético ou peróxido de hidrogénio, envelhecimento cronológico, ou pela expressão heteróloga de proteínas Bax *c*-myc) e a localização de Aif1p foi avaliada por microscopia de fluorescência e fracionamento celular /western Blot. No entanto, não fomos capazes de observar libertação de Aif1p para o citosol ou a sua importação nuclear nas condições experimentais utilizadas. Tentámos também mapear o domínio NLS de Aif1p através da remoção de vários domínios homólogos com domínios conhecidos de AIF humano. Embora não tenhamos ainda conseguido mapear o domínio NLS do Aif1p, descobrimos que a deficiência no domínio putativo de ligação a Hsp70 resulta numa localização aberrante de Aif1p, sugerindo que a Hsp70 é importante para a estabilidade desta proteína e que esta proteína pode ainda estar envolvida na regulação da localização de Aif1p na levedura.

Index

Abbreviations

AIF – Apoptosis Inducing Factor
AIFL - AIF-Like
ANT - Adenine Nucleotide Translocator
AMID - AIF homologous mitochondrion-associated inducer of death
Apaf-1 - Apoptotic Protease Activating Factor-1
ARM - Arginine-rich motifs
ATP - Adenosine Triphosphate
CARD - Caspase Recruitment Domain
c-FLIP - Cellular-FLICE (FADD-like IL-1β-converting enzyme)-inhibitory Protein
Caspases - Cysteine-dependent Aspartate-specific Proteases
Cyp A - Cyclophilin A
Cyt *c* – Cytochrome *c*
DAPI - 4,6-Diamino-2-phenyl-indole dihydrochlorid
DD - Death Domain
DED - Death Effector Domain
DISC - Death Inducing Signaling Complex
DNA - Deoxyribonucleic Acid
DR - Death Receptors
EDTA - Ethylenediaminetetraacetic acid
Endo G - Endonuclease G
ER - Endoplasmatic Reticulum
FADD - Fas-Associated Death Domain
GDP - Guanosine Diphosphate
GFP - Green Fluorescent Protein
GTP – Guanosine triphosphate
h - hours
H_2O_2 – Hydrogen Peroxide
HSPs - Heat-shock proteins
HtrA2/Omi - High Temperature Requirement Protein A2
IAPs - Inhibitors of Apoptosis Proteins
IBB - Importin-β binding
IMM - Inner mitochondrial membrane
IMS - Intermembrane space
Kaps - Karyopherins
MAC - Mitochondrial Apoptosis-induced Channel
MOMP - Mitochondrial Outer Membrane Permeabilization
NADH - Nicotinamide Adenine Dinucleotide
NADPH - Nicotinamide Adenine Dinucleotide Phosphate
min - Minutes
MLS – Mitochondrial Localization Sequence
NE – Nuclear envelope
NES - Nuclear Export Sequence
NLS - Nuclear Localization Sequence
NF-KB - Nuclear factor kappa-light-chain-enhancer of activated B cells
NPC - Nuclear pore complexes
Nups – Nucleoporins
OD – Optical density
OMM - Outer mitochondrial membrane
PCD – Programmed Cell Death
PCR - Polymerase Chain Reaction
phe-gly - phenylalamine-glycine
PTP - Permeability Transient Pore
ROS - Reactive Oxygen Species
rpm – Rotations per minute
sec - Seconds
Smac/Diablo - Second mitochondria-derived Activator of Caspases/Direct Inhibitor of Apoptosis Protein (IAP)-Binding Protein With Low Pi
SPB - Spindle Pole Body
TNF-R - Tumor-Necrosis Factor Receptor
TRADD - TNF-R-Associated Death Domain
TRAIL-R - TNF-related Apoptosis-Inducing Ligand Receptor
VDAC - Voltage-Dependent Anion Channel

1. INTRODUCTION

1.1. Apoptosis

Multicellular organisms often need to eliminate cells that are in excess, or damaged cells that can put the organism at risk (Hengartner, 2000). To this end, they use a highly regulated and complex process characterized by a group of endogenous molecular events that culminates in “cell suicide” (Leist *et al.,* 2001), designated by programmed cell death (PCD). Several types of cell death have been associated with PCD, but in eukaryotic cells the most common is apoptosis (Hengartner, 2000). Apoptosis occurs during normal development of multicellular organisms and continues through adult life. This process is as important as cell division or cell migration because it allows the organism to control cell number and tissue size, as well as protect itself from compromised cells that can threaten homeostasis, such as infected or damaged cells (Gewies, 2003).

The term apoptosis derives from the Greek “apo” - from and “ptosis” – falling, an analogy to the term used by Greeks to describe leaves falling from trees. This term was used in 1972 by John Kerr and colleagues to describe a type of cell death with distinct morphologic characteristics (Lawen, 2003). Indeed, apoptotic cells can easily be recognized by several morphological features such as cell shrinking, chromatin condensation and migration along the nuclear membrane, blebbing of the plasma membrane and exposure of phosphatidylserine to the outer leaflet of the plasma membrane. The final hallmark of apoptosis is cell fragmentation into compact structures, called 'apoptotic bodies' that will be phagocyted by macrophages and eliminated from the tissue without leading to an inflammatory response (Saraste *et al.,* 2000).

1.1.1. Mechanism of apoptosis

Apoptosis can be triggered by various stimuli and mechanisms, including virus infection, cell stress and DNA damage. Cellular sensitivity to these stimuli can differ depending on several factors, such as expression of anti- and pro-apoptotic proteins, the harshness of the stimulus and the phase of the cell cycle (Gewies, 2003). The apoptotic process consists of three consecutive steps: (i) a trigger by extracellular or intracellular stimuli; (ii) execution by activation of intracellular proteases and (iii) elimination of dead cells by engulfment of cell debris by neighboring cells or macrophages (Saikumar *et al.*, 1999). Deficient regulation of apoptosis can lead to various pathologies (Rudin and Thompson, 1997); improper activation may cause or contribute to several diseases such as ischemic strokes, AIDS (acquired

immunodeficiency syndrome), and several neurodegenerative disorders (Raff *et al.*, 1993, Ameisen *et al.*, 1995 and Smale *et al.*, 1995). In contrast, a flawed activation of this process can lead to some autoimmune diseases and to uncontrolled cell division that culminates in the development of cancers (Tan, 1994; Lowe and Lin, 2000 and Reed, 2003).

Two major apoptotic pathways have been identified in mammalian cells: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Figure 1) (Hengatner 2000 and Elmore, 2007).

1.1.1.1. Extrinsic pathway

In the extrinsic pathway, "death receptors" are activated to transmit apoptotic signals from the exterior to the interior of cells. Examples of death receptors are TNFR-1, Fas/CD95 and TRAIL receptors. Death receptors belonging to the tumor necrosis factor receptor (TNFR) gene superfamily are present in the cell surface and transmit the apoptotic signal after ligation of precise ligands such as Fas ligand, TNF alpha and TRAIL. They have an important role in apoptosis because they can trigger a caspase cascade within seconds of ligand binding and induce apoptosis very quickly (Sartorius *et al.*, 2001).

Caspases (Cysteine-dependent Aspartate-specific Proteases) are conserved through evolution and can be found in several organisms such as insects, human, hydra and nematodes (Cohen, 1997). They are synthesized as inactive zymogens, named procaspases. They have a prodomain followed by a small and a large subunit that are occasionally separated by a linker peptide. After maturation, procaspases are proteolytically processed between the large and the small subunit. An active caspase is a heterotetramer that consists of two large and two small subunits (Hengartner, 2000). Their active site contains a cysteine residue critical for catalytic activity; it has high affinity for aspartate residues, after which caspases cleave their substrates. So far, about a hundred caspases substrates have been described (Fischer *et al*., 2003).

There are two different groups of caspases: (i) initiator caspases, which include the procaspases -2, -8, -9 and –10, with long prodomains such as death effector domains (DED) in procaspase-8 and -10 or caspase recruitment domains (CARD) in procaspases-9 and -2; (ii) effector or executioner caspases, with only short prodomains, which include procaspases -3, -6 and -7 (Chrowdhury *et al.*, 2008).

In the extrinsic pathway, initiation of apoptosis requires both caspases and death receptors. Apoptotic signaling is mediated by the death domain (DD) of the death

receptor. Adapter molecules that possess their own DDs, as FADD or TRADD, are then recruited to the DDs of the death receptor to form the death-inducing signaling complex (DISC). With the help of FADD, procaspase 8 (initiator procaspase) is then recruited to the DISC, and the amount of procaspase-8 molecules in the DISC leads to their autocatalytic activation and consequent release of active caspase-8. Active caspase-8 then activates the caspase signaling cascade; subsequently, effector caspases cleave specific substrates, resulting in cell death (Stegh *et al.*, 1998; Scaffidi *et al.*, 1998). It is possible to inhibit caspase-8 in order to protect cells from apoptosis. One example is cellular-FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP), which can inhibit DISC signaling. This inhibition will lead to inactivation of DISC and by consequence inhibition of the extrinsic pathway (Hengartner, 2000). In some cases, the apoptotic signal produced by death receptors might not be capable of inducing a caspase signaling cascade strong enough to lead to cell death on its own. In other cases, there may be a bridge between the caspase signaling cascade and mitochondria provided by Bid, a Bcl-2 family member (see below). Bid can be cleaved into its truncated form (tBID) by caspase-8 and translocated to the mitochondria, where it induces the release of pro-apoptotic proteins to the cytosol (Luo *et al.*, 1998 and Gustafsson *et al.*, 2007).

1.1.1.2. Intrinsic pathway

The intrinsic pathway is triggered by “intrinsic” stresses such as DNA damage, endoplasmic reticulum stress, lysosomal stress, and mitochondrial dysfunction. In contrast with the extrinsic pathway, in which caspases are activated directly, the intrinsic pathway requires the participation of mitochondria. Permeabilization of the outer mitochondrial membrane (MOMP) and the release of pro-apoptotic factors are crucial events of the intrinsic pathway and required for activation the downstream caspase cascade. Mitochondria have thus gained great importance in the field of mammalian apoptosis, since they are able to amplify the apoptotic signal from the extrinsic pathway and also propagate the death signals generated within the cell, such as oxidative stress, DNA damage and others (Wang, 2001).

The Bcl-2 family, a group of apoptotic regulators with both anti- and pro-apoptotic members, plays a major role in the regulation of the intrinsic pathway. Bcl-2 family members share homologous regions known as BH domains, and can be divided into four categories based on structural and functional similarities, namely: i) the anti-apoptotic Bcl-2 proteins (A1, Bcl-2, Bcl-w, Bcl-xL and Mcl-1); ii) Bcl-2 effector proteins

(Bax and Bak); iii) direct activator BH3-only proteins (Bid, Bim and Puma) and iv) sensitizer/derepressor proteins (Bad, Bik, Bmf, Hrk and Noxa) (Chipuk *et al.*, 2010). Pro- and anti-apoptotic proteins can interact with each other and with several other proteins, forming several regulatory networks, and is the balance of the levels of pro- and anti-apoptotic proteins that can dictate the fate of the cell, mainly through regulation of MOMP.

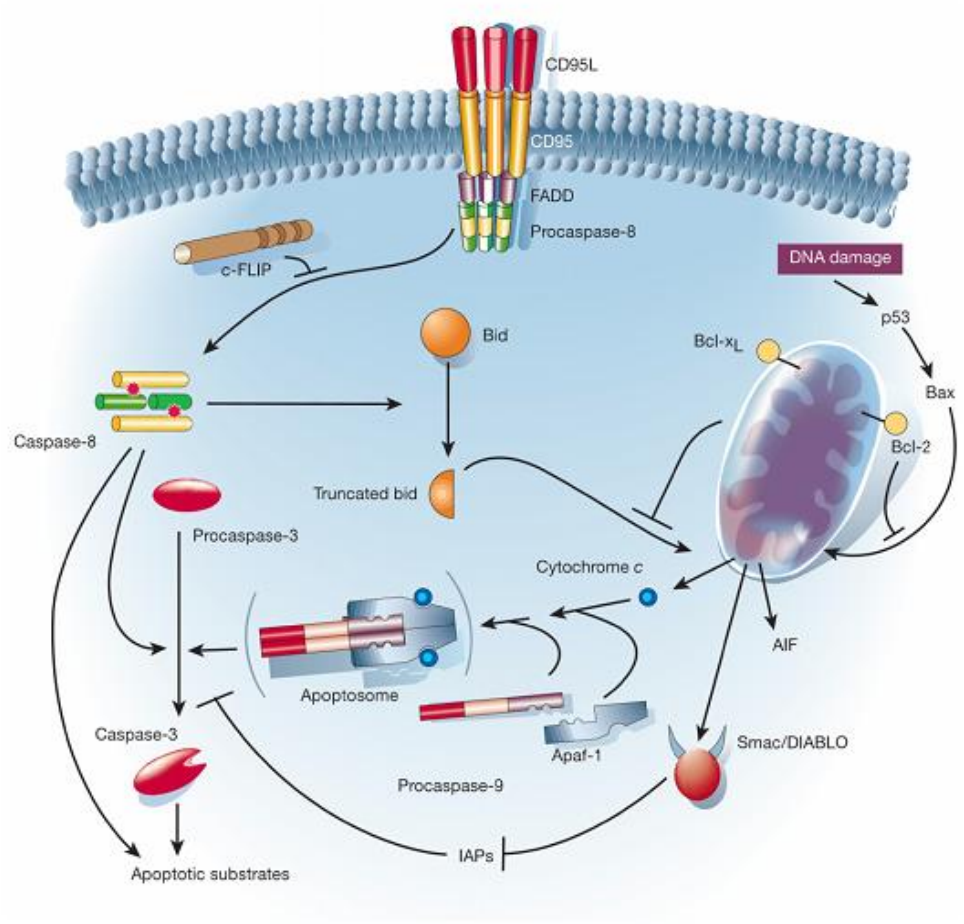


Figure 1 - Schematic representation of the extrinsic and intrinsic pathways involved in apoptosis (Hengatner 2000).

Mitochondria are surrounded by two membranes, the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane (OMM), which divide the mitochondria into two compartments: the intermembrane space (IMS) between the membranes, and the inner compartment called the matrix. Many stress stimuli can trigger permeabilization and release of pro-apoptotic proteins from the mitochondria to the cytosol. Two main hypotheses explaining how permeabilization of mitochondria

occurs have been put forth. The first hypothesis suggests that a transmembrane channel named the permeability transition pore (PTP) is formed on the contact sites between the IMM and the OMM. These pores are thought to be mainly constituted by VDAC in the OMM and by adenine nucleotide translocator (ANT) in the IMM. PTP opening results in loss of membrane potential, uptake of solutes and entry of water to the matrix, leading to the rupture of the outer membrane and release of pro-apoptotic proteins from the IMS to the cytoplasm (Lawen, 2003). Another hypothesis is based on the ability of the pro-apoptotic Bcl-2 proteins to form pores. Indeed, the accepted model proposes a stepwise structural reorganization of Bax leading to mitochondrial targeting and homo-oligomerization. Bax is kept as a monomeric soluble cytosolic factor through the engagement of its α9 helix in the dimerization pocket by the α1 helix. The activator BH3s, tBID/BIM/PUMA, attack and expose the α1 helix of Bax, leading to a secondary disconnection of the α9 helix and consequently mitochondrial insertion. Activator BH3s stay associated with the N-terminally exposed Bax through the BH1 domain to drive homo-oligomerization and activation of Bax (Ren *et al.*, 2010).

Permeabilization of mitochondria results in the release of several proteins such as cytochrome *c*, Omi/HtrA2 (High Temperature Requirement Protein A2) and Smac/DIABLO (Second Mitochondria-derived Activator of Caspases/Direct Inhibitor of Apoptosis Protein (IAP)-binding Protein With Low pI). Later, the apoptosis inducing factor (AIF) and endonuclease G (EndoG) are also released. All these proteins are located in the mitochondrial intermembrane space (IMS) and are released to the cytosol in response to several apoptotic stimuli, such as extra- and intracellular stresses like oxidative stress and treatment with cytotoxic drugs (Vaux, 2011). Proteins of the first group activate the caspase-dependent mitochondrial pathway. After its release to the cytosol, cytochrome *c* can interact with Apaf-1 (Protease Activating Factor – 1), leading to the recruitment of pro-caspase 9, which together with dATP forms a multi-protein complex, the apoptosome, which in turn activates caspase -9 leading to apoptosis. On the other hand, Smac and Omi/HtrA2 normally neutralize IAP (Inhibitors of Apoptosis Proteins), which normally inhibit effector caspases. Pro-apoptotic proteins of the second group are released only after the cell has committed to die. Both AIF and Endo G are translocated to the nucleus (Joza *et al.,* 2001). AIF induces DNA fragmentation and chromatin condensation, whereas Endo G induces DNA internucleossomal fragmentation (Wang, 2001).

1.2. Apoptosis inducing factor (AIF)

The mammalian mitochondrial AIF is a flavoprotein that belongs to a larger family of proteins with common structural and functional features, containing bacterial, plant and fungal oxidoreductases. It is a protein with 613 aminoacids and is structurally divided into three typical domains: a FAD and a NADH binding domain and a C-terminal domain, where its pro-apoptotic activity resides. In addition, it also possesses an N-terminal MLS (Mitochondria Localization Signal) (Figure 2) (Gurbuxani *et al.*, 2003). Besides AIF, AIF homologous mitochondrion-associated inducer of death (AMID) and AIF-Like (AIFL) also belong to this family (Hangen *et al.*, 2010).

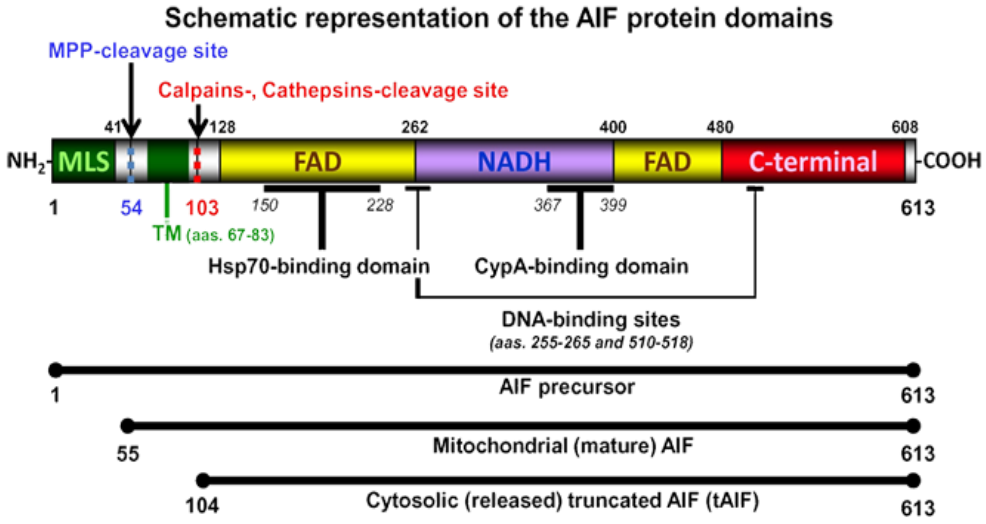


Figure 2 – Representation of the three different AIF forms: the precursor, the mature and the truncated AIF. AIF contains an N-terminal MLS domain (green), a FAD bipartite domain (yellow), an NADH binding domain (violet) and a C-terminal domain (red). It also possesses an Hsp70p and a Cyclophilin A binding domain. Cleavage at the MPP (Mitochondrial Processing Peptidase) cleavage site (blue dotted line) generates mitochondrial mature AIF and cleavage by calpains (at the red dotted line) generates truncated AIF (tAIF) (Yuste *et al*., 2007).

In healthy cells, AIF is confined to mitochondria, where it plays an important role in bioenergetic and redox metabolism. However, after an apoptotic stimulus, AIF is released from mitochondria to the cytosol and translocated into the nucleus (Modjtahedi *et al.*, 2006).

1.2.1. The role of AIF in cells

AIF has dual functions, in DNA fragmentation and in redox activity, and thus acts both in cell death and cell survival. However, biochemical and mutational analysis of AIF suggests that its apoptotic and redox functions can be separated (Lipton *et al.*, 2002). Three distinct functions have been associated with AIF, as seen in figure 3.

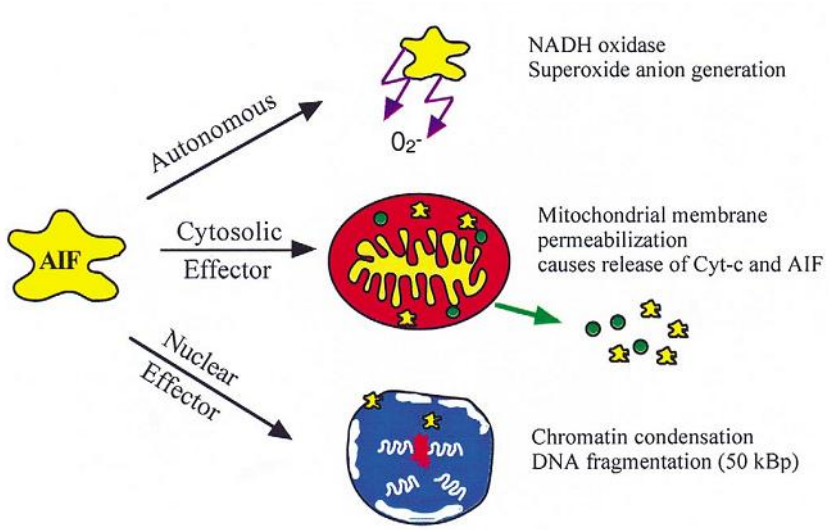


Figure 3 - Representation of the three different functions of AIF in cells. First, AIF has a role as an NADH oxidase. Second, cytosolic AIF seems to promote mitochondrial membrane permeabilization and third, AIF can promote chromatin condensation and DNA fragmentation (Candé *et al.*, 2002).

The first function attributed to AIF is it NADH oxidase activity. AIF has a redox potential of –308 mV ± 15 mV at pH 7.5. It has monodehydroascorbate reductase activity and catalyses cytochrome *c* reduction in the presence of NADH. Results have shown that the AIF NADH oxidase activity requires an electron donor (NADH), an electron acceptor and a prosthetic FAD group for catalytic electron transfer (Miramar *et al.*, 2001).

The second function is permeabilization of the mitochondrial membrane and consequent release of cytocrome *c* and additional AIF. This function is attributed to cytosolic AIF, since studies revealed that when AIF is introduced into the cytosol it can induce the release of AIF and cytochrome *c* from mitochondria (Daugas *et al.*, 2000). When cells commit to apoptosis, AIF translocates to the nucleus, where it exerts its third function: to trigger cell death by promoting chromatin condensation and DNA fragmentation (Candé *et al.*, 2002). Several reports suggest that this role of AIF in the

nucleus functions as a backup to caspase-dependent mechanisms. However, it was also described that AIF-induced cell death occurs in the complete absence of caspases and the oxireductase activation. AIF seems to play a role in caspase-independent PCD in several organisms such as *Caenorhabditis elegans*, *Dyctiostelium discoideum* and mammals (Arnoult *et al.*, 2001, Wang *et al.*, 2007, Wang *et al.*, 2002 and Lorenzo *et al.*, 2007).

But how are these effects achieved at the molecular level? The crystal structure of AIF shows that the surface of the protein has positively charged amino acids, allowing it to form electrostatic connections with DNA. AIF binds with more affinity to linearized forms than to intact circular plasmids, suggesting that it is introduced into the DNA strand breaks. The AIF-DNA interaction is accompanied by DNA condensation (shortening of the DNA), hairpin formation (intermolecular packageing) and DNA oligomerization (Modjtahedi *et al.*, 2006). Studies have shown that addition of AIF to DNA does not cause DNA degradation. It was therefore suggested that after entering the nucleus AIF can cooperate with several exo- and endonucleases, building up a so-called “degradeosome” including cyclophilins, proteins belonging to the family of peptidylprolyl cis–trans isomerases. Human AIF is able to interact with cyclophilin A (CypA) and forms an active DNase, and several studies confirm that CypA is essential for the apoptotic activity of AIF (Candé *et al.*, 2004). These characteristics can be included into a hypothetical model: AIF invades DNA strand-breaks, induces DNA condensation and finally participates in the formation of a DNA-degrading polyprotein complex (Figure 4). It is still not known if the deadly effect of the translocation of AIF to the nucleus is due to inhibition of the respiratory chain or to apoptotic DNA degradation in the nucleus.

It has recently been discovered that AIF acts in programmed necrosis, in addition to its involvement in apoptotic PCD. PCD is a dynamic process that depends on the characteristics of the involving scenario, such as cell or tissue type, death stimulus or environmental conditions. Cells can use caspase or non-caspase dependent mechanisms, exhibiting necrotic or apoptotic features. Programmed necrosis is thus an active capase-independent pathway. Moubarak *et al.* described that AIF is a regulator of this type of PCD by causing extensive DNA damage when cells are exposed to high doses of MNNG, an alkylating agent and necrotic inducer. In this study, AIF knockout protected cells from DNA damage and cell death, highlighting the requirement of this protein for programmed necrosis (Moubarak *et al.,* 2007, Boujrad *et al.*, 2007 and Schabel, 1976).

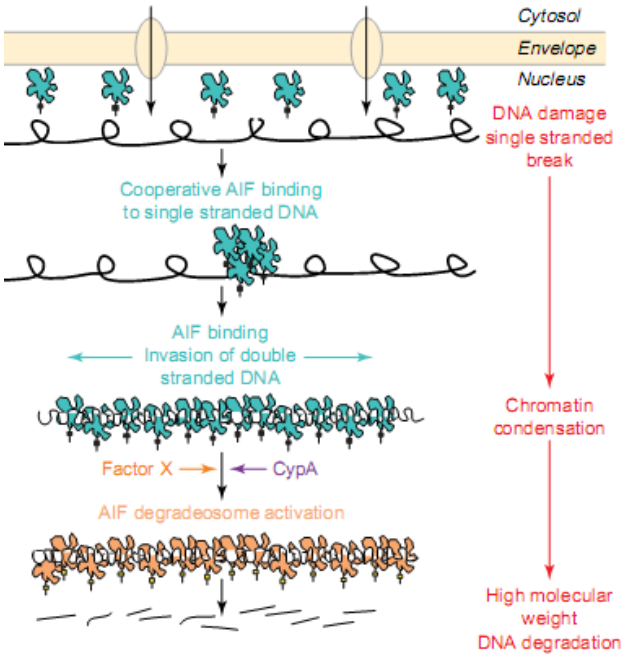


Figure 4 - Hypothesized model for the action of AIF on DNA (Modjtahedi *et al*, 2006).

1.2.2. The mechanism underlying AIF release from mitochondria

AIF is encoded by a nuclear gene and is synthesized in the cytoplasm. It is imported to mitochondria through the general import pathway as a 67 kDa protein with a mitochondrial localization signal (MLS) in the N-terminus and anchored to the IMM. Upon import to the mitochondria IMS, the MLS is cleaved by a mitochondrial peptidase to generate mature 62 kDa AIF. As referred above, after treatment with certain apoptotic stimuli, AIF can be cleaved from its membrane anchor by proteases (calpains and cathepsins), forming a soluble 57 kDa AIF fragment (Figure 2). Calpains are a family of calcium-dependent cysteine proteases. There are two main isoforms, calpain-I and calpain-II, which co-exist with the calpain-specific inhibitor calpastatin. The interaction between the two prevents both activation and catalytic activity of calpains. Their localization is uncertain, but recently calpain was found to contain an MLS and it was suggested that calpains are present in the mitochondrial IMS. Upon activation, calpains can cleave and destroy calpastatin that, with addition of Ca^{2+} to the mitochondria, is sufficient to stimulate AIF cleavage, as illustrated in figure 5 (Badugu *et al.*, 2008 and Norberg *et al.*, 2010).

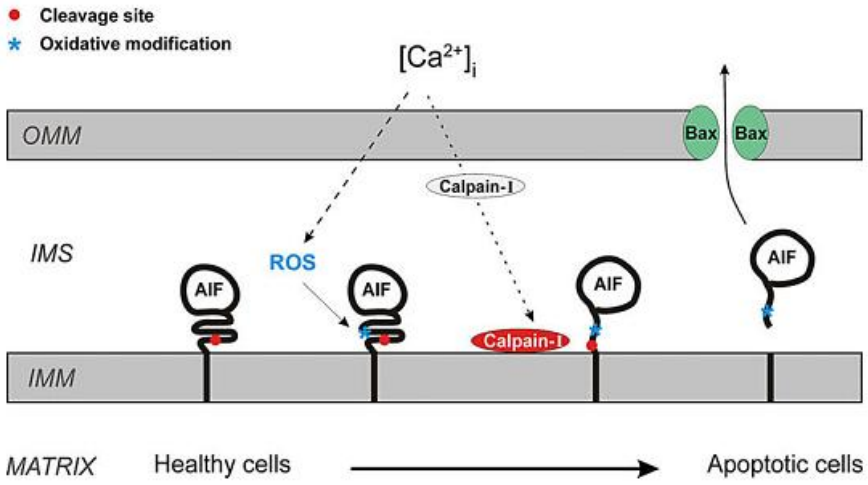


Figure 5 - Schematic representation of the mechanism behind the release of AIF from IMM to the cytoplasm (Norberg *et al.*, 2010).

1.2.3. Heat shock protein 70 (Hsp70p) acts as a chaperone to AIF protein

After permeabilization of the OMM, cleaved AIF can be released to the cytosol and translocated to the nucleus. However, this translocation seems to be regulated by cytoprotective proteins. The first proteins thought to play this role were heat-shock proteins (HSPs). This group of proteins is found in most the living organisms, such as, bacteria, yeast, humans, and others. They are involved in the folding and unfolding of other proteins and their expression increases when cells are exposed to several stresses but mostly when cells are exposed to elevated temperatures (Parsell and Lindquist, 1993). This family of chaperones recognizes a broad spectrum of unfolded or misfolded proteins (Chakrabart *et al.*, 2011). Using a series of biochemical tests, it was discovered that, within the family of HSPs proteins, Heat-shock protein 70 (Hsp70p) was the only capable of physically interacting with AIF. Hsp70p is involved in the inhibition of apoptosis by blocking Apaf-1 and by consequence the formation of the apoptosome, (Ravagnan *et al.*, 2001). It also binds to and neutralizes AIF, protecting cells against translocation of AIF to the nucleus and by consequence protecting against its apoptogenic effects, such as chromatin condensation and fragmentation. This is corroborated by several studies, where the authors demonstrate that the interaction between AIF and Hsp70p inhibits its translocation to the nucleus both in cultured cells and *in vivo* (Ravagnan *et al.*, 2001 and Ruchalski *et al.*, 2001). However, the precise

mechanism of AIF import into the nucleus is not known. Yeast Aif1p shows the same localization and exhibits similar death executing pathways as mammalian AIF. Therefore, this model system is particularly suitable to investigate the mechanism involved in importing AIF to the nucleus.

1.3. Yeast apoptosis

Over the last decades, *Saccharomyces cerevisiae* has been a preferred research tool in numerous areas of cell biology, due to its easy handling, technical tractability, fast growth, small sequenced genome and the fact it is a eukaryote. However, it was often questioned whether single-cell organisms need to commit suicide or die by apoptosis. In fact, yeast cells tend to form colonies and communities, and it is hypothesized that apoptosis may occur in yeast during chronological and replicative ageing, leading to removal of virus-infected and damaged cells and unsuccessful mating processes from colonies. This altruistic cell death gives younger cells nutrients they can metabolize, contributing to the maintenance of the members of the community (Büttner *et al*., 2006) (Figure 6). It has now become clear that apoptosis does not occur only in multicellular organisms, but can also be triggered in unicellular organisms like yeast. It is believed that several apoptotic pathways are conserved in yeast, making this organism attractive to study programmed cell death (Carmona-Gutierrez *et al.*, 2010).

The first observations of yeast apoptosis were made in a mutant strain of *S. cerevisiae* with a mutation in the *CDC48* gene, which encodes a protein necessary for vesicle trafficking. The authors observed that dying cells of this mutant showed an apoptotic phenotype with several characteristics of mammalian apoptosis, such as phosphatidylserine exposure to the outer leaflet of the plasma membrane, DNA fragmentation and margination, and condensation of chromatin (Madeo *et al.*, 1997).

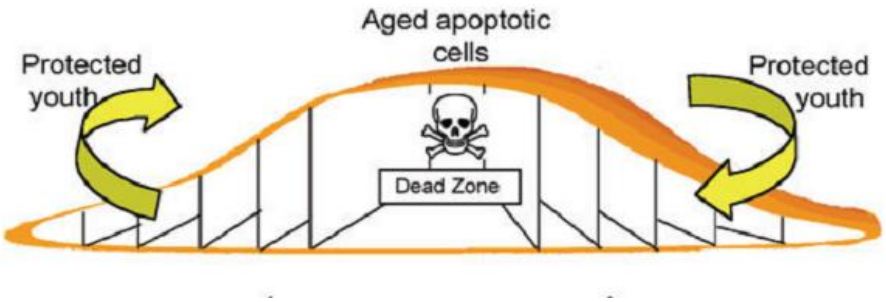


Figure 6 – Representative image of how cell death and ageing occurs in a yeast colony. The amount of aged cells located in the center of the colony corresponds to the dead zone. These cells die altruistically to give to the younger cells located at the periphery nutrients, allowing the maintenance of the colony (adapted from Gourlay *et al*., 2006).

Since then, multiple yeast orthologs of mammalian apoptotic proteins have been found and characterized. They include AIF, IAP, caspases, AMID, Endo G and Omi/HtrA2 (Madeo *et al.*, 2004). In addition, cell death has been associated with chronological and replicative ageing and cell cycle arrest (Madeo *et al.*, 2002).

Another similarity of yeast apoptosis with mammalian apoptosis is the important role of mitochondria in both processes, through ROS production and release of pro-apoptotic proteins from the mitochondria IMS. Moreover, mitochondrial morphology is radically reorganized during apoptosis, from filamentous tubules into small punctate spheres. This dynamic nature of mitochondrial morphology is due to two processes, fission and fusion, important for maintaining mitochondrial function and repair damaged mitochondria. The morphological change of mitochondria seems to be related with cell death, as one of the early markers of mammalian apoptosis is fragmentation of tubular mitochondria. In yeast, there are three genes involved in mitochondrial fission which have been associated with regulation of apoptosis: *DNM1*, *MDV1/NET2* and *FIS1*. Deletion of the first two (*DNM1, MDV1/NET2*) protects cells from death induced by acetic acid or Hydrogen peroxide (H_2O_2) treatment, whereas deletion of the latter (*FIS1*) leads to an increase in cell death. This observation suggested that Fis1p could have a function similar to that of Bcl-2 proteins (Fanjiang *et al.*, 2004). However, it was recently found that the sensitivity of *fis1*Δ mutants to cell death is due to a secondary mutation in the stress-response gene *WHI2* (Cheng *et al*., 2006), which confers a sensitive phenotype in response to apoptosis inducers.

Like mammals, yeast also has caspase-dependent and caspase-independent apoptotic pathways. *YCA1*, the yeast orthologue of mammalian caspases, is

considered a metacaspase and has a central role in yeast apoptosis. It is known that yeast cells with a disruption of *YCA1* are less susceptible to apoptotic cell death under stress conditions like ageing and exposure to oxidative stress, salt, valproic acid, iron and other metals. Recently, another caspase-like protease in yeast was found, Eps1p. When released from the anaphase inhibitor Pds1p, Eps1p works as a caspase-like protease and cleaves Mcd1p. Mcd1p is a yeast homolog of human cohesion Rad21 that is truncated after apoptotic stimuli and translocates from the nucleus to the mitochondria, decreasing the mitochondrial membrane potential and thus promoting the release of cytochrome *c* (Madeo *et al.*, 2009). However, not all yeast apoptotic cell death is dependent on *YCA1* or on another caspase-like protease.

1.3.1. Yeast apoptotic triggers

In yeast, apoptosis can be triggered by three different strategies: by heterologous expression of pro-apoptotic proteins, by environmental stress or drug-induced stress, or by yeast endogenous apoptotic inducers.

Heterologous expression of proteins consists in expressing one or various proteins in an organism that does not possess these proteins in its own genetic background. It is described that yeast cells do not possess obvious orthologous of proteins from the Bcl-2 family. However, heterologous expression of these human pro-apoptotic proteins can trigger an active cell death. While some studies showed that when Bax or Bak proteins are expressed in yeast, they can trigger cell death with apoptotic characteristics, such as release of cytochrome *c* and generation of ROS, others demonstrated that Bax induces a cell death process associated with activation of autophagy (Kissova *et al*., 2007). These facts suggest that these proteins have their function conserved in this organism (Eisenberg *et al.*, 2007 and Madeo *et al.,* 2004).

It is also known that several exogenous agents such as hyperosmotic stress, heavy metals, amiodarone, ethanol, elevated temperatures, oxidative stress, UV radiation, various pharmacological agents (such as aspirin), osmotin, viral "killer" toxins, HOCl, pheromones and sometimes sugar can efficiently induce apoptosis in yeast (Liang *et al.*, 2008; Silva *et al.*, 2005; and Carmona Gutierrez *et al.,* 2010). However, the best characterized and the most used exogenous inducers of apoptosis in yeast are H_2O_2 and acetic acid (Madeo *et al.*, 1999 and Ludovico *et al.*, 2001).

Finally, several endogenous factors that trigger yeast apoptosis have been described, such as defects in cellular processes like chromatid cohesion, N-glycosylation, mRNA stability (Carmona-Gutierrez *et al.*, 2010). Other examples of

endogenous apoptosis triggers are DNA damage resultant from oxygen metabolism and ROS generation and failed replication. Chronological and replicative ageing are additional examples, and apoptosis also occurs during the development of colonies on solid media (Herker *et al.*, 2004 and Vachova and Palkova, 2005). Other endogenous triggers are some pro-apoptotic proteins that are released from the mitochondria to the cytosol, such as AIF, AIF-homologous mitochondrion-associated inducer of death (AMID) and EndoG orthologues, which once in the cytosol are translocated to the nucleus.

1.3.2. Yeast orthologue of AIF, Aif1p

Aif1p, the yeast orthologue of AIF, was first described in 2004 by Wissing *et al*. It was shown that Aif1p, as mammalian AIF, is mitochondrial in normal cells and relocalizes to the nucleus in the presence of apoptotic stimuli. It was also found that the role of yeast Aif1p is dependent on cyclophilin A and partially on caspase activity. Therefore in yeast, in contrast with mammalian cells, Aif1p-mediated apoptosis is not completely independent on the yeast metacaspase Yca1p. Moreover, in contrast to mammalian AIF, purified Aif1p degrades yeast nuclei and plasmid DNA (Wissing *et al.*, 2004).

AIF1 deletion led to increased survival to treatment with H_2O_2 and acetate, as well as to decreased chronological ageing. Like in mammalian cells, Aif1p also has a vital role in respiration via its NADH oxidase domain, and *AIF1*-deficient *S. cerevisiae* have decreased growth on non-fermentable carbon sources. This redox function is important for an effective anti-oxidant defense and oxidative phosphorylation (Madeo *et al.*, 2009).

1.3.2.1. Stimuli that regulate Aif1p release

Ludovico (2002) was the first to observe translocation of Aif1p to the nucleus in cells undergoing chronological ageing or treated with camptothecin, an S phase-specific anticancer drug that inhibits the action of the enzyme DNA topoisomerase-I. Then Wissing *et al.* (2004) also observed translocation of Aif1p to the nucleus, in cells treated with H_2O_2. Since then, few additional studies have addressed this issue and the role of Aif1p in yeast apoptosis. Morton *et al.* described that antimicrobial peptides, the dermaseptins obtained from amphibians, are capable of inducing yeast cell death. The yeast cell death described by this group is independent of the metacaspase Yca1p but

depends on Aif1p for nuclear fragmentation (Morton *et al.*, 2007). Bostrycine is an anthracenedione with antimicrobial and phytotoxic activity that belongs to the quinone family, which inhibits cell proliferation and induces a decrease in the mitochondrial membrane potential, leading to mitochondrial disruption. When yeast cells were treated with this compound, cell death with hallmarks of apoptosis was observed. However, bostrycin-induced cell death was promoted in *yca1*Δ. In contrast, this death phenotype was partially rescued in *aif1*Δ cells (Chunlingku *et al.*, 2009). Another substance that can induce apoptosis is allicin, an antimicrobial extracted from garlic that is capable of inducing apoptosis through its oxidative properties; both Aif1p and Yca1p seem to be involved in this process, suggesting that allicin induces apoptosis through an alternative mechanism (Gruhlke *et al.*, 2010).

So far only H_2O_2 and chronological ageing have been shown to trigger Aif1p release to the cytosol and translocation to the nucleus (Wissing *et al.*, 2004). However, there have been no studies addressing the mechanism underlying the import of yeast Aif1p into the nucleus, which, like for mammalian AIF, is most likely an active and highly regulated process.

1.4. Nucleocytoplasmic trafficking

The nucleus is a defining characteristic of eukaryotic cells and is physically separated from the cytosol by the nuclear envelope (NE), a double membrane structure. Most genetic information is confined to the nucleus, where most functions of the cell are thus governed. Non-cytosolic proteins synthesized in the cytoplasm have to be directed to their specific locations, where they exert their functions. For that they contain a signal sequence that will direct them to a specific location. For example, proteins containing a nuclear localization sequence (NLS) are imported into the nucleus (Macara, 2001).

1.4.1. Nuclear Pore Complex (NPC)

The NE is crossed by multiple supramolecular structures named nuclear pore complexes (NPCs), structures specialized in transport of small molecules, ions and macromolecules between the cytoplasm and the nucleus. The number of NPCs in each cell is variable and depends on cell size and transcriptional activity, and differs between species. Human cells may contain about $5x10^3$ – $5x10^7$ NPCs per nucleus and these NPCs have about 60 MDa of molecular weight. The yeast NPC is much smaller than

that of higher eukaryotes, with a molecular weight of approximately 44 MDa, and there are about 200 NPCs per nucleus (Freitas *et al.*, 2009).

NPCs consist of a cylindrical central body sandwiched between the outer and inner layers of the NE. Nucleoplasmic and cytoplasmic rings are constituted by eight subunits that constitute the central structure. Eight protein filaments rise up from the cytoplasmic ring towards the cytoplasm, and the nuclear ring forms eight filaments that converge in a structure named nuclear basket, as shown in figure 7.

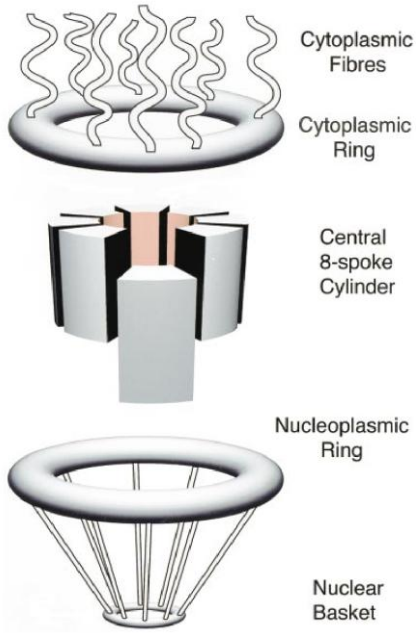


Figure 7 - Representation of a human nuclear pore complex (NPC) showing its morphology (Bayliss *et al.*, 2000).

The central structure of the human NPC has a central channel about 30 nm in diameter that allows the transport of ions and proteins with a molecular weight of 20-40 kDa (Bayliss *et al.*, 2000). Some proteins can be transported into the nucleus by an intrinsic ability to directly interact with the NPC, but most are transported through the NPC by association with several types of transport receptors. The overall structure of the NPC is similar between human and yeast, but yeast NPCs lack the cytoplasmic and nuclear rings (Rout *et al.*, 2000). Amazingly, despite their large size, purified yeast NPCs contain only about 30 different proteins, named nucleoporins (Nups). Nups can be separated into three groups: nucleoporins that span the nuclear membrane; non-membrane proteins that have multiple repeats of a phenylalamine-glycine (phe-gly)

motif, and non-membrane proteins that don't have the phe-gly motif (Macara *et al.*, 2001).

1.4.2. Protein transport receptors and transport cycle

For the majority of macromolecules, transport into the nucleus and through the NPCs is an energy-dependent process. This process can be mediated by the major class of transport receptors, which includes different soluble proteins (karyopherins or Kaps, also named importins, exportins or transportins). They mediate import into or export from the nucleus of proteins that cannot be transported by simple diffusion through the NPCs, as well as mediate the transport of non-coding RNAs. Karyopherins are acidic proteins with a molecular weight of 90 to 145 kDa. About 10 of the 20 karyopherins identified were demonstrated to participate in the nuclear import of proteins in eukaryotes (Tran *et al.*, 2006). Sequence analysis identified 13 predicted importin-β proteins in the yeast *S. cerevisiae*.

Most transport receptors of the importin-β family are capable of binding directly to cargo and transport it across the NE while others, like importin-β, need an adaptor. Importin-β can interact with importin-α and mediate a process of transport into and out of the nucleus. Importin-α is a transport adaptor that recognizes and binds to the NLS of proteins. The NLS-binding domain is formed by 10 arginine-rich motifs (ARM) in tandem, localized on the central region of importin-α. The NLS of the cargoes is recognized by importin-α through ARM, and the interaction between the importins is mediated by an importin-β binding domain (IBB) that is localized in the N-terminal region of importin-α. The cargo/importin-α/importin-β complex is then transported through the NPCs. In the nucleus, this complex is dissociated by binding of Ran-GTP to importin-β, which displaces the cargo. Importin-α is then transported back to the cytosol by the exportin Cse1/CAS (Figure 8) (Cook *et al.*, 2007). However, most karyopherins are able to directly interact with their cargo and recognize a variety of nuclear localization signals, though most are basic in nature.

Another important characteristic of karyopherins is that they can interact with Ran GTPase and the phe-gly domains of nucleoporins. Ran GTPase, a monomeric protein belonging to the Ras superfamily, can be found in two distinct forms; GTP-bound and GDP-bound, and these two different forms are distributed asymmetrically in the nucleus (more Ran-GTP) and in the cytoplasm (more Ran-GDP). Specific regulatory proteins that are localized in the cytoplasm and the nucleus maintain the asymmetric distribution of these proteins. Normally, the reaction of hydrolysis of GTP

into GDP is slow; however, this reaction can be accelerated by two proteins, Ran-GAP and Ran-BP1. On the other hand, the nuclear protein RCC1 promotes the reverse reaction. It is the combined action of Ran regulatory proteins that creates and maintains the Ran-GTP gradient across the NE, and it is this gradient that establishes the directionality of nucleocytoplasmic transport (Freitas *et al.*, 2009).

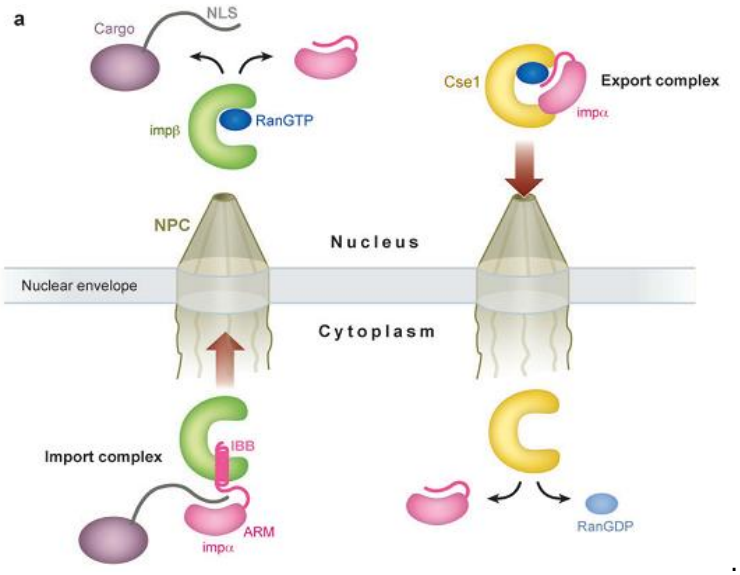


Figure 8 - Overview of the transport cycle of the classical nuclear localization signal (NLS) transport with the ligation of importin-β and importin-α (Cook *et al.*, 2007).

Ran-GTP binds to the transport receptors (importin-β family) and also to other proteins in solution. Ran-GTP, in the nucleoplasmic side of the NPC, dissociates import receptors from their cargo, promoting release of the cargo. In contrast, it promotes the formation of export complexes. Ran-GAP and Ran-BP1, located in the cytoplasm, promote hydrolysis of Ran-GTP bound to export complexes, releasing the cargo and freeing the carrier for another round of transport (Figure 9).

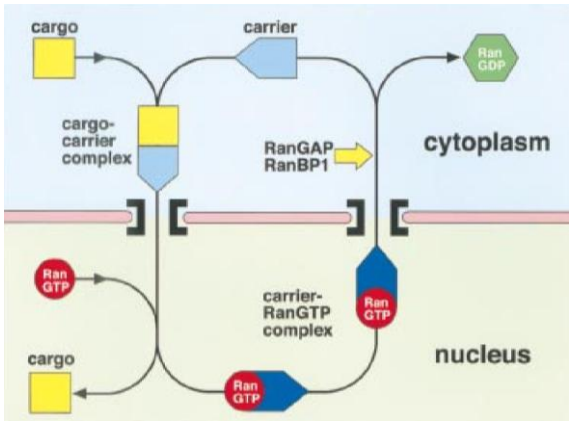


Figure 9 - Schematic representation of processes underlying nuclear import and export mediated by β-karyopherins (Importin-β) (Bayliss *et al.*, 2000).

Several cycles of export complexes with Ran-GTP would lead to depletion of Ran from the nucleus. Ran is small and can therefore diffuse through the NPC, but this mechanism is not fast enough for efficient nuclear trafficking (Bayliss *et al.*, 2000). It is known that the nuclear import of Ran-GDP is promoted by the nuclear transport factor NTF2/p10. After import of Ran-GDP to the nucleus, the protein RCC1 will convert the Ran-GPD into Ran-GTP (Figure 10) (Cook *et al.*, 2007**,** Freitas *et al.*, 2009). The levels of Ran-GTP are thus replenished in the nuclear side, allowing for continuous cycles of import and export.

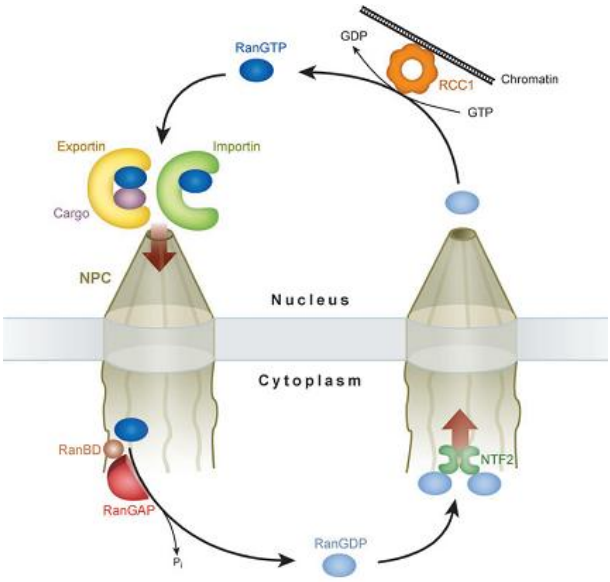


Figure 10 - Schematic representation of the Ran cycle (Cook *et al.*, 2007).

1.4.3. Regulation of nucleocytoplasmic trafficking

As mentioned above, the majority of proteins are synthesized in the cytosol and then transported to their final localization in order to exert their function. This process is often an important regulatory step in cellular pathways. Indeed, there are several examples of proteins whose function is regulated by nuclear localization. One example is NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), a transcription factor found in almost all types of mammalian cells. It is involved in the cellular response to several stimuli, such as several types of stress, cytokines, ultraviolet radiation, free radicals and LDL oxidation. This transcription factor is bound to an inhibitory protein (IkB) in unstimulated cells and therefore is latent. However, upon activation by extracellular agents, IkB is phosphorylated by a protein kinase and then ubiquitinated and degraded by the proteasome, allowing NF-kB translocation to the nucleus. There, it binds to the kB consensus sequence generally leading to an increase in the expression of the target gene (O'Neill *et al.*, 1997).

Another example is the yeast transcription factor Yap1p, which regulates expression of target genes containing a binding site named Yap1p-response element on their promoter. Normally Yap1p is located in the cytosol and is translocated into the nucleus under stress conditions. In the nucleus, nuclear export of Yap1p is inhibited, which leads to its accumulation in the nucleus and consequently inducing the expression of several genes encoding antioxidant proteins. Exposure to H_2O_2 induces the formation of disulfide bonds between the C-terminal cysteine-rich domain (C-CRD) and the N-terminal cysteine-rich domain (N-CRD) of Yap1p. The C-CRD possesses a NES, which ordinarily leads to export of Yap1p from the nucleus, but it is masked by this dually disulfide-bonded structure. Therefore, Yap1p export is inhibited, promoting Yap1p nuclear accumulation (Gulshan *et al.*, 2005).

Another example of regulated transport is the regulation of protein localization by phosphorylation and desphosphorylation of the yeast transcription factor Pho4p. Pho4p localization is regulated in response to modifications in the concentration of inorganic phosphate in the media. Pho4p is imported into the nucleus through the importin-β family member Pse1p/Kap121p. It is known that inhibition of the Pho4p/Pse1p interaction is mediated by phosphorylation and that Pho4p translocation requires Pse1p. This suggests that this import is regulated by phosphorylation *in vivo*. In yeast cells grown in phosphate-rich medium, Pho4p is phosphorylated by the Pho80-Pho85 cyclin-CDK complex and import into the nucleus is therefore inhibited. Consequently, there is no transcription of phosphate starvation-specific genes. However, in yeast grown with limited phosphate, the CDK inhibitor Pho81p inhibits

Pho80-Pho85, leading to accumulation of unphosphorylated Pho4p. Unphosphorylated Pho4p can then interact with Pse1p/Kap121p, translocate into the nucleus and induce transcription of phosphate-responsive genes (Kaffman *et al.*, 1998).

1.4.4. Nuclear import of AIF

AIF function is regulated at different levels including through its subcellular localization. As mentioned above, AIF is transported to the mitochondria due to the N-terminal MLS. After mitochondrial membrane permeabilization, truncated AIF is released into the cytosol and then translocated to the nucleus.

The crystal structure of AIF indicates that this protein has to be re-localized to the nucleus to exert its apoptotic activity (Ye *et al.*, 2002). To discover which region of this gene is responsible for the apoptotic activity of this protein, Gurbuxani and colleagues mapped functional regions of AIF by deleting several regions and observing the resultant phenotype. The authors determined that the C-terminal domain (beyond residue 567) is responsible for AIF-induced chromatin condensation (once AIF is in the nucleus). Deletion of the Hsp70p-binding region (residues 150 to 228) leads to a gain of function phenotype, *i.e.*, facilitates nuclear translocation in response to an apoptotic stimulus. One region of AIF contains a consensus NLS (residues 367 to 459), normally involved in protein translocation to the nucleus. However, two NLS domains are described for AIF, one more N-terminal (residues 277 to 301) and another closer to the C-terminus (residues 445–451) (Susin *et al.*, 1999). In this study, the authors showed that the C-terminal NLS is functionally more important for AIF translocation to the nucleus than the N-terminal NLS domain. However, deletion of the region containing the C-terminal NLS only partially inhibited AIF translocation, which suggests that there are other domains involved in AIF import (Gurbuxani *et al.*, 2003). However, the mechanism mediating this nuclear translocation of AIF is still unclear.

2. AIMS AND RESEARCH PLAN

Apoptosis-inducing factor is a flavoprotein with oxidoreductase activity localized in the mitochondrial intermembrane space. Upon apoptosis induction, AIF translocates to the nucleus, where it leads to chromatin condensation and DNA degradation. AIF has been suggested to control a caspase-independent pathway of apoptosis, important for neurodegeneration and normal development. However, it remains unknown how AIF translocates to the nucleus. Yeast Aif1p shows the same localization and exhibits similar death executing pathways as mammalian AIF, though it mediates a partially caspase- dependent pathway (Wissing *et al.,* 2004). Therefore, our aim was to use the yeast model system to investigate the mechanism involved in importing Aif1p to the nucleus.

Aim 1. Determine which soluble import factor is necessary to import Aif1p

Transform mutants in each of the yeast soluble transport receptors (Kaps) with Aif1p-GFP and assess the localization of Aif1p after apoptosis induction by fluorescence microscopy. Mutation of the Kap mediating Aif1p import will prevent its nuclear accumulation.

Aim 2. Map the NLS of Aif1p

Clone different fragments of Aif1p in frame with GFP and assess their localization by fluorescence microscopy. Identify the smallest sequence that is both necessary and sufficient for transport into the nucleus.

3. MATERIALS AND METHODS

3.1. Plasmids

All the plasmids and oligonucleotides used in this work are listed in Table I and II, respectively. Plasmids were amplified in the *Escherichia coli* XL1Blue strain (as described below) and purified using a Miniprep kit (GenElute Plasmid Miniprep kit, Sigma-Aldrich) according to manufacturer's instructions. The identity of the plasmids was confirmed by digestion with specific restriction enzymes and by PCR.

pAIF1WT-GFP, pAIF1ΔMLS-GFP, pAIF1ΔHSP70-GFP, pAIF1ΔNLS-GFP were constructed by Gap Repair (Figure 11). Briefly, four fragments of the *AIF1* gene (Wild-type AIF1, AIF1 ΔMLS, AIF1 ΔHSP70, AIF1 ΔNLS) were amplified by Polymerase Chain Reaction (PCR) using the plasmid pAIF1 from Euroscarf as a template and oligonucleotides forward 1-4, respectively, and the reverse oligonucleotide 5 (Table II). *Saccharomyces cerevisiae* strain DF5a was transformed with pYX242GFP vector digested with *EcoR*I and *Hind*III and each of the four fragments (Figure 11). Correct integration of the *AIF1* fragments in pYX242GFP was confirmed by colony PCR using oligonucleotides that anneal upstream and downstream of the insertion (6 and 7, respectively). After confirmation, the plasmids were purified from yeast cells (described below in 3.6), amplified in *E. coli* and sequenced.

Table I – List of the plasmids used in this work.

Plasmid	Description	References/Sources
pAIF1	*AIF1* ORF	Euroscarf (Germany)
pYX242GFP	*LEU2*, *AmpR*	Rosenblum *et al*., 1998
pAIF1WT-GFP	*AIF1* 1-1137 cloned in pYX242GFP	This study
pAIF1ΔMLS-GFP	*AIF1* Δ1-78 cloned in pYX242GFP	This study
pAIF1ΔHSP70-GFP	*AIF1* Δ1-336 cloned in pYX242GFP	This study
pAIF1ΔNLS-GFP	*AIF1* Δ1-723 cloned in pYX242GFP	This study
pBAX *c*-myc	Bax *c*-myc cloned in PCM189, *URA3*	Priault *et al.*, 1999

Table II – List of the oligonucleotides used in this work.

Number	Name	Oligonucleotide sequence (5' - 3')
1	AIF1WT Fw	ATCTATAACTACAAAAAACACTATCAGGAATTCGGGCCCATGACA
2	AIF1ΔMLS Fw	ATCTATAACTACAAAAAACACTATCAGGAATTCGGGCCCATGAGGG AACTGGGT
3	AIF1ΔHSP70 Fw	ATCTATAACTACAAAAAACACTATCAGGAATTCGGGCCCATGCCA
4	AIF1ΔNLS Fw	ATCTATAACTACAAAAAACACTATCAGGAATTCGGGCCCATGGGT
5	AIF1 Rev	GACAACAACAGTGAATAATTCTTCACCTTTAGACATCCGGGG
6	YX Fw	ATCTATAACTACAAAAAACACATACAGGAATTCGGGCCCATGACA
7	GFP Rev	AGCGTCGACGTTACCTTATTTGTACAATTCATCCATACCATGGG
8	YX Fw	CTTTTTAATTCTAAATCAATCTTTTTCAA
9	GFP Rev	AACATCACCATCTAATTCAAC

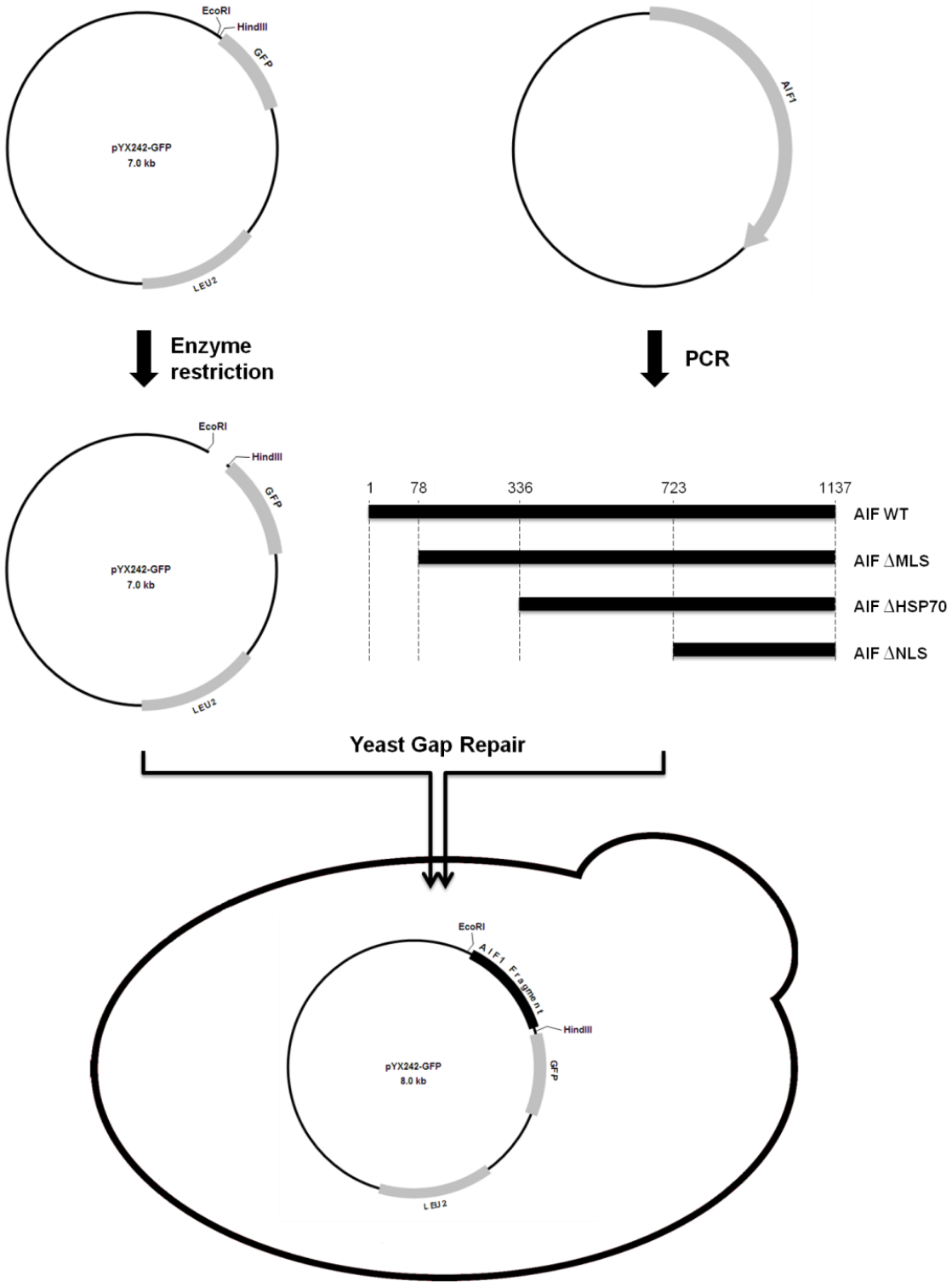


Figure 11 - Schematic representation of the methodology used in the amplification of *AIF1* fragments (AIF1 ΔMLS, AIF1 ΔHSP70, AIF1 ΔNLS) and construction of plasmids.

3.2. Yeast strains and growth conditions

All *S. cerevisiae* strains used in this work and respective genotypes are shown in Table III. Strains were transformed with the indicated plasmids using the Lithium acetate/Single Stranded carrier DNA/Polyethylene Glycol (PEG) method previously described in (Gietz and Woods, 2006). Transformants were selected on Synthetic Complete (SC) medium [SC containing 0.17% (w/v) Yeast nitrogen base without aminoacids and ammonium sulfate, 0.5% (w/v) ammonium sulfate, 0.14% (w/v), drop-out mixture lacking histidine, leucine, tryptophan and uracil, 0.008% (w/v) Histidine, 0.04% (w/v) Leucine, 0.008% (w/v) Tryptophan and 0.008% (w/v) Uracil] lacking the appropriate aminoacids plus 2% (w/v) of carbon source and 2% agar. Yeast strains were maintained on solid YPD or SC medium (lacking the appropriate amminoacids), grown at 30°C for 48 h, stored at 4°C, and refreshed every 2 weeks. Yeast cultures were grown aerobically in SC medium with 2% Glucose or Galactose as a carbon source. Strains transformed with plasmids were grown in the same medium lacking the appropriate amino acids. Cells were incubated at 30°C with orbital shaking (200 rpm) and a liquid/air ratio of 1:5.

3.3. Hydrogen peroxide and acetic acid treatment

Cells were grown overnight until exponential phase (OD_{600} = 0.5-0.6) on SC Glu, collected by centrifugation and resuspended in new medium (medium with pH 3 in the case of acetic acid treatment) to a final concentration of 10^7 cells/mL (approximately OD_{600} = 0.2) and incubated with H_2O_2 (1 mM, 2 mM and 3 mM), or acetic acid (140 mM, 160 mM, 180 mM) for up to 360 min at 30°C. At specific time points, serial dilutions (1:10) were spotted onto YPD plates and colony growth was scored after 2 days of incubation at 30°C. Viability was determined in relation to time 0 (100%). In parallel, 500 µL of cells were harvested and processed for fluorescence microscopy.

3.4. Heterologous expression of Bax *c*-myc

Strains harboring the plasmid pBAX *c*-myc were grown overnight in SC Glucose lacking uracil and supplemented with doxycycline (10 µg/ml) to repress Bax *c*-myc expression. Cells were centrifuged, washed three times with sterilized water, ressuspended in the same medium without doxycycline (to induce Bax *c*-myc expression) or with doxycycline (as a negative control). At specific time points, serial

dilutions (1:10) were spotted onto YPD plates and colony growth was scored after 2 days of incubation at 30°C. Viability was determined in relation to time 0 (100%). In parallel, 500 µL of cells were harvested and processed for fluorescence microscopy.

Table III – List of *S. cerevisiae* strains used in this work.

Strain	Genotype	Reference/Source
DF5a	Mat a*;* ura3-52, leu2-3, 112 lys2-801, trp1-1, his3Δ200	Finley *et al*., 1987
BY4741	Mat a; his3Δ 1, leu2Δ 0, met15Δ 0, ura3Δ 0	Euroscarf (Germany)
BY4742	Mat α; leu2, lys2, ura3, his3	Euroscarf (Germany)
W303a	Mat a; ade2; ura3; his3; trp1; leu2	Costa V., IBMC
*ssa1*Δ	BY4741 YAL005C :: *KanMX4*	Euroscarf (Germany)
DF5a pYX242GFP	DF5a harboring pYX242GFP	This study
DF5a pAIF1WT-GFP	DF5a harboring pAIF1WT-GFP	This study
DF5a pAIF1ΔMLS-GFP	DF5a harboring pAIF1ΔMLS-GFP	This study
DF5a pAIF1ΔHSP70-GFP	DF5a harboring pAIF1ΔHSP70-GFP	This study
DF5a pAIF1ΔNLS-GFP	DF5a harboring pAIF1ΔNLS-GFP	This study
W303 pAIF1WT-GFP	W303 harboring pAIF1WT-GFP	This study
W303 pAIF1ΔMLS-GFP	W303 harboring pAIF1ΔMLS-GFP	This study
W303 pAIF1ΔHSP70-GFP	W303 harboring pAIF1ΔHSP70-GFP	This study
W303 pAIF1ΔNLS-GFP	W303 harboring pAIF1ΔNLS-GFP	This study
W303 pAIF1WT-GFP pBAX *c*-myc	W303 harboring pAIF1WT-GFP and pBAX *c*-myc	This study
BY4741 pAIF1WT-GFP	BY4741 harboring pAIF1WT-GFP	This study
BY4741 pAIF1ΔMLS-GFP	BY4741 harboring pAIF1ΔMLS-GFP	This study
BY4741 pAIF1ΔNLS-GFP	BY4741 harboring pAIF1ΔNLS-GFP	This study
BY4741 pAIF1WT-GFP pBAX *c*-myc	BY4741 harboring pAIF1WT-GFP and pBAX *c*-myc	This study
BY4741 pAIF1ΔMLS-GFP pBAX *c*-myc	BY4741 harboring pAIF1ΔMLS-GFP and pBAX *c*-myc	This study
*ssa1*Δ pAIF1WT-GFP	*ssa1*Δ harboring pAIF1WT-GFP	This study
*ssa1*Δ pAIF1ΔMLS-GFP	*ssa1*Δ harboring pAIF1ΔMLS	This study
*ssa1*Δ pAIF1WT-GFP pBAX *c*-myc	*ssa1*Δ harboring pAIF1WT-GFP *and* pBAX *c*-myc	This study
*ssa1*Δ pAIF1ΔMLS-GFP pBAX *c*-myc	*ssa1*Δ harboring pAIF1ΔMLS-GFP and pBAX *c*-myc	This study

3.5. Transformation of bacterial cells

Super Optimal Broth medium (250 ml) [SOB; 2% (w/v) Tryptone peptone; 0.5% (w/v) Yeast extract; 2.5 mM KCl, 10 mM NaCl; 10 mM $MgSO_4$, 10 mM $MgCl_2$] was inoculated with 2 colonies of XL1Blue *E. coli* strain and grown at 18°C, 200 rpm with a ratio of flask liquid/air of 1:5 until OD_{600} = 0.6, about 4-5 days. Then the culture was placed on ice for 10 min and the cells pelleted for 10 min at 2500g, at 4°C. The pellet was suspended in 80 mL ice-cold TB buffer [10 mM Pipes; 15 mM $CaCl_2$; 250 mM KCl; 55 mM $MnCl_2$] and left on the ice for 10 min. Cells were centrifuged for 10 min at 2500 g and gently suspended in 20 mL ice-cold TB buffer. DMSO was added to a final concentration of 7% and cells incubated on ice for 10 min. Finally, competent cells

were aliquoted, frozen with liquid nitrogen and stored at -80°C. *E. coli* transformation was performed by a standard protocol for chemically competent cells and transformants selected on Luria Bertani medium [LB; 1% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 1% (w/v) NaCl and 2% (w/v) Agar] with 100 µg/mL of Ampicillin.

3.6. Purification of Yeast DNA

Plasmid DNA from yeast cells was purified using the GenElute Plasmid Miniprep kit (Sigma-Aldrich) according to manufacturer's instructions, with modifications. Cells from a 10 mL overnight culture were centrifuged and the pellet resuspended in 200 µL of Resuspension solution. About 200 µL of glass beads and 10 µL of lyticase were added to the cells and the tubes incubated for 10-20 min at 37°C. Next, tubes were vortexed for 15 min. Cells were lysed by adding 200 µL of Lysis solution and gentle inversions of the tubes. 350 µL of Neutralization solution was added and the tubes inverted 5-6 times, and the lysate spun at 15000 rpm for 10 min. The columns from the kit were washed with 500 µL of Column Preparation solution, and the supernatant from the neutralization step was loaded into the columns after centrifugation at 15000 rpm for 1 min, columns were washed with 500 µL of Wash Optional solution, then with 750 µL of Wash solution and dried. The columns were changed to new tubes, and DNA eluted with 50 µL of Elution buffer.

3.7. Fluorescence Microscopy

Samples (200 µL) were harvested and centrifuged for 2 min at 10000 rpm, and the pellet was resuspended in 100 µL of Phosphate-Buffered Saline [PBS; 80 mM Na_2HPO_4, 20 mM NaH_2PO_4 and 100 mM NaCl], fixed in 100% (v/v) ethanol and 4 µL of DAPI (2 µg/mL) added. After 5 min of incubation, cells were washed twice with PBS and resuspended in 10 µL of PBS. Cells were visualized in a Leica Microsystems DM-5000B epifluorescence microscope using appropriate filter settings with a 100x oil immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

3.8. Subcellular fractionation

3.8.1. Preparation of spheroplasts

Cells were grown in 2.4 L of SC medium without appropriate aminoacids and using Galactose as the carbon source, to obtain more mitochondrial mass. For acetic

acid treatment, cells were grown until late exponential phase (OD_{600} = 1) and the culture divided in two: one culture, without treatment, was used as a negative control and the other was treated with 120 mM of acetic acid (pH 3,0) for 120 min. For Bax *c*-myc expression, cells were treated as described above, in the presence and absence of doxyclyclin. Cells were collected and incubated in Pre-incubation buffer [0.5 M *β*-mercaptoethanol, 0.1 M Tris, pH 9.3] for 15 min at 30°C and washed 2 times with Tris/KCl buffer [10 mM Tris, 0,5 M KCl, pH 7]. Spheroplasts were obtained by incubation in Digestion buffer [1.35 M Sorbitol, 10 mM Citric acid, 30 mM Na_2PO_4, 1 mM EDTA, pH 5.8] containing 10 mg or 50 mg of zymoliase /1 O.D for untreated and treated cells. Digestion of cell wall was performed during 30 and 50 min for untreated and treated cells, respectively.

3.8.2. Mitochondrial and cytosolic fraction preparation

Spheroplasts were washed with Washing buffer [0.750 M Sorbitol, 0.4 M Mannitol, 10 mM Tris Maleate, pH 6.8] and disrupted by osmotic shock in Homogenization buffer [0.6 M Mannitol, 10 mM Tris Maleate, pH 6.8] and mild homogenization in a hand-potter, to preserve the outer mitochondrial membrane. The homogenate was centrifuged at 2500 rpm to remove cell debris and nuclei, and the supernatant centrifuged at 15000 rpm for 15 min. The pellet corresponds to the mitochondrial fraction and the supernatant to the cytosolic fraction. Mitochondria were then resuspended in Recuperation buffer [0.6 M Mannitol, 2 mM EDTA, 10 mM Tris Maleate, pH 6.8].

3.8.3. Nuclear fraction preparation

3.8.3.1. Protocol I

Crude nuclei resulting from the 2500 rpm centrifugation were washed 3 times with Recuperation buffer (3000 g for 5 min) and the final washed pellet resuspended in Recuperation buffer and loaded onto a Ficoll 400 step gradient in Ultra-Clear tubes (30, 40 and 50% (w/v)). The gradient was centrifuged at 18000 rpm for 60 min at 2°C. The nuclei band in the 40% layer was collected. Tubes containing the nuclear extract were stored at -80°C.

3.8.3.2. Protocol II

Spheroplasts were resuspended in half volume of Nuclei buffer [1 M Sorbitol, 20 mM PIPES pH 6.3, 0.5 mM $CaCl_2$, 1 mM DTT, 0.1 M EDTA, 1 M PMSF] and added dropwise to an erlenmeyer containing Ficoll buffer [18% (w/v) Ficoll 400, 20 mM PIPES pH 6.3, 0.5 mM $CaCl_2$, 1 mM DTT, 0.1 M EDTA, 1 M PMSF] with continuous agitation, in a ice-cold bath to prevent protease activity. The Ficoll suspension was centrifuged at 5000 rpm for 5 min at 4°C, and this step was repeated until the supernatant become clear. The supernatant was then transferred to new tubes and the tubes centrifuged at 13000 rpm for 20 min at 4°C. The pellet was resuspended in 5-10 volumes of Nuclei buffer and the suspension centrifuged again at 9000 rpm for 10 min at 4°C. The final pellet (nuclear fraction) was resuspended in Storage buffer [20 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 100 mM KCl, 10% (v/v) Glycerol] and stored at -80°C.

3.8.3.3. Protocol III

The third protocol used was based on protocol II, but only serial centrifugations were performed. The protocol started with two washes at 500 rpm of the crude nuclei resultant from the 2500 rpm centrifugation, followed by centrifugations at 1000, 2000, 6000, 9000 and 13000 rpm of the supernatants. All centrifugations were performed during 10 min and all the washes and resuspensions were made with recuperation buffer. Samples of the sedimented pellets in each centrifugation were collected to determine the sedimentation pattern of the different organelles.

3.8.4. SDS gel electrophoresis/Westernblot

Protein concentration of the fractions was estimated through the Bradford method using BSA as standard (Bradford, 1976). Proteins (50 µg) of the subcellular fractions were separated electrophoretically on a 15% SDS polyacrylamide gel at 20 mA and transferred to a Hybond-P Polyvinylidene Difluoride Membrane (PVDF) (Hybond-ECL, GE Healthcare) at 60 mA for 1 h and 30 min. Membranes were blocked for 1 h in PBS-T [PBS with 0.05% (v/v) Tween-20] containing 5% (w/v) non-fat dry milk, washed in PBS-T and then cut into strips and incubated with the primary antibodies; mouse monoclonal anti-yeast phosphoglycerate kinase (PGK1) antibody (1:5000, Molecular Probes), mouse monoclonal anti-yeast porin (POR1) antibody (1:5000, Molecular Probes), rabbit polyclonal anti-yeast cytochrome *c* (CYC1) antibody (1:1000, custom-made by Millegen), mouse monoclonal anti-green fluorescent protein (GFP)

antibody (1:3000; Roche Applied Science), rabbit polyclonal anti-histone H4 (H4B) antibody (1:2000, abcam), rabbit polyclonal anti-human Bax (BAX) antibody (1:10000, Sigma). Then membranes were incubated with secondary antibodies against mouse or rabbit IgG-peroxidase (1:10000; Sigma Aldrich). Pgk1p and Por1p were used as controls for cytosolic and mitochondrial fractions, respectively. Immunodetection of bands was revealed by chemiluminescence (Immobilon, Millipore).

3.9. Immunoprecipitation

Immunoprecipitation was performed using protein G-coupled Dynabeads (Invitrogen). Briefly, cytosol was prepared as described above (See 3.8.2), supplemented with a mixture of protease inhibitors (0.4 µL/mL Aprotinin, 1 µg/mL Leupeptin, 1 µg/mL Pepstatin, 1 mM Phenylmethylsulfonyl fluoride) and with 100 mM NaCl and 0.01% NP-40. 1, 2 or 5 µg of monoclonal anti-GFP antibody (Roche Applied Science) were added and the lysate was incubated overnight at 4°C with agitation. Protein G-coupled Dynabeads (30 µL) were then added and incubated for 6 h at 4°C with agitation. Beads were washed 6 times for 2 min with IP buffer [50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 2 mM EDTA, 0.01% (v/v) NP-40] and bound proteins eluted with Laemmli sample buffer for 5 min at 95°C. The sample buffer was collected to new tubes.

3.9.1. SDS gel electrophoresis/Western Blot/ Silver Staining of immunoprecipitated proteins

Immunoprecipitated samples were loaded in duplicate onto a 4-20% Mini-PROTEAN TGX Precast Gel (BioRad) to guarantee a good separation of proteins. One half of the gel was transferred to PVDF as described above and Aif1p-GFP detected using mouse monoclonal anti-green fluorescent protein (GFP) antibody as the primary antibody (1:3000; Roche Applied Science).

The other half of the gel was stained by silver staining. The gel was fixed by washing at least twice with Fixing Solution [30% (v/v) Ethanol, 10% (v/v) Acetic Acid] for 30 min. After fixing, the gel was washed with Washing Solution [30% (v/v) Ethanol] for 10 min and with deionized water for 10 min. The gel was then soaked with Sensitizing Solution [0.02% (w/v) Sodium Thiosulfate] and carefully agitated for 1 min and rinsed three times with deionized water for 30 sec. The gel was then incubated with gentle agitation with freshly made Staining Solution [0.15% (w/v) Silver Nitrate] for at least 30 min, washed 3 times with deionized water and transferred to Developing

Solution [0.05% (v/v) Formaldehyde, 3% (w/v) Sodium carbonate]. When an adequate degree of staining was achieved, gels were transferred to Stop Solution [5% (v/v) Acetic acid] for 1 min and then placed in deionized water.

4. RESULTS

4.1. Construction of pAIF1WT-GFP plasmid and localization Aif1p

Aif1p is synthesized in the cytosol and imported into mitochondria, where it acquires its mature form. In response to certain stimuli, Aif1p is released to the cytosol and then translocated into the nucleus, where it promotes chromatin condensation and DNA fragmentation (Norberg *et al.,* 2010). However, the mechanism involved in the mobilization of Aif1p between these cellular compartments is not completely understood. Our goal was therefore to determine which yeast cytosolic factors are required for this nuclear relocalization. First, we aimed to determine which apoptotic stimuli triggered this relocalization more efficiently. In order to assess the localization of Aif1p, we constructed a plasmid expressing Aif1p in frame with the green fluorescent protein (GFP) by gap repair (pAIF1WT-GFP). We then assessed the localization of Aif1p-GFP in living cells by fluorescence microscopy (Figure 12). Aif1p-GFP clearly localized to the tubular mitochondria network.

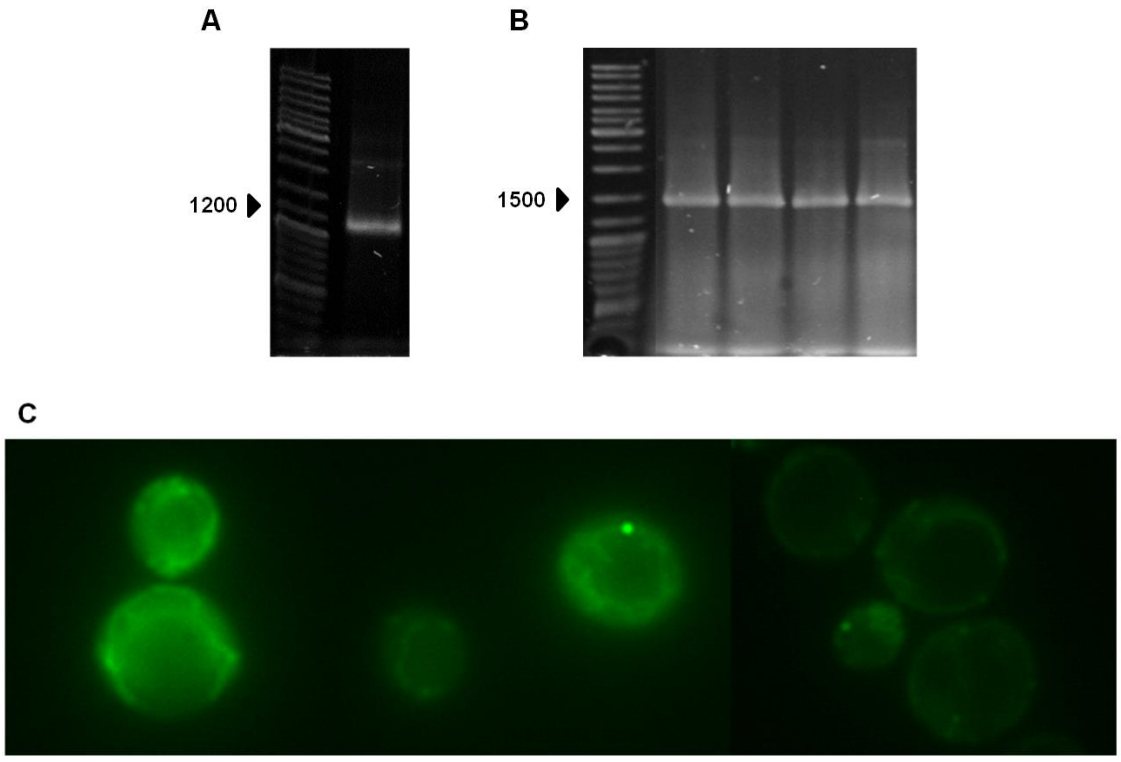


Figure 12 – The construction of the plasmid was achieved in the DF5a strain through Gap Repair. *AIF1* was amplified by PCR using as template the pAIF1 from Euroscarf and oligonucleotides 1 and 5 (see Table 2). The vector used was pYX242GFP digested with *EcoR*I and *Hind*III.PCR confirmation gel of *AIF1* amplification **(A)** Colony PCR was performed in four candidates using oligonucleotides 5 and 6 to confirm the correct insertion of *AIF1* into the plasmid (see Table 2) **(B).** Localization of Aif1p-GFP in the W303 strain by fluorescence microscopy **(C).**

It has been described that yeast Aif1p is translocated from the mitochondria to the nucleus in response to several stimuli, including exogenous stimuli such as chemical stresses (H_2O_2 or acetic acid), or endogenous stimuli triggered by chronological ageing (Gutierrez *et al.,* 2010). Therefore, cells expressing pAIF1WT-GFP plasmid were exposed to these different apoptosis-inducing conditions. Heterologous expression of human pro-apoptotic proteins, such as the pro-apoptotic member of the Bcl-2 family Bax, triggers permeabilization of the outer mitochondrial membrane (MOMP) and the subsequent release of cytochrome *c*. Therefore, we also addressed whether under this latter condition of MOMP induction, Aif1p is also released and translocated to the nucleus.

4.2. Acetic acid treatment and chronological ageing

Exposure of *S. cerevisiae* to acetic acid at pH 3 leads to cell death with features of mammalian apoptosis, namely exposure of phosphatidylserine, DNA strand breaks and chromatin condensation (Ludovico *et al.*, 2001). Moreover, cell death induced by acetic acid is associated with the mitochondrial pathway and results in cytochrome *c* release, ROS production and other mitochondrial dysfunctions (Ludovico *et al.*, 2002). The length of time that a yeast cell can survive in a non-dividing state is called yeast chronological lifespan (Fabrizio and Longo, 2003). Chronological ageing has been described as a physiological trigger for apoptosis in yeast (Herker *et al.*, 2004; Fabrizio and Longo, 2003). Consistently, Wissing *et al.* observed nuclear localization of Aif1p in chronologically aged cells.

We first attempted to observe Aif1p translocation to the nucleus using these two stimuli to induce apoptosis. Acetic acid treatment was performed as previously described. Cells expressing pAIF1WT-GFP were treated with 140 mM, 160 mM, 180 mM of acetic acid for up to 360 min or grown for 3-4 days for chronological ageing studies, as described by Wissing *et al.* (2004) and cells were observed by fluorescence microscopy. However, we could observe that there was a complete loss of fluorescence in cells after acetic acid treatment and chronological ageing, in comparison with cells from T0 (data not shown). This may be due to the intracellular acidification of cells, which occurs in both cases (Sokolov *et al.*, 2006; Fabrizio *et al.,* 2004). As accumulation of Aif1p-GFP in the nucleus had been previously observed in cells undergoing acetic acid-induced cell death and in aged cells (Wissing *et a.l*, 2004), we hypothesize that the GFP variant used in that study might have been less sensitive to pH changes.

4.3. Hydrogen peroxide treatment

Hydrogen peroxide (H_2O_2) is a reactive oxygen species (ROS) described as an apoptotic stimulus at low doses (Madeo *et al.*, 1999). Wissing *et al.* (2004) described that Aif1p-GFP translocates from the mitochondria to the nucleus when cells are exposed to H_2O_2. We therefore next attempted to observe Aif1p translocation to the nucleus using H_2O_2 as an apoptotic stimulus. W303 cells expressing pAIF1WT-GFP were grown and treated with 1 mM and 3 mM of H_2O_2 for up to 360 min, stained with DAPI for nuclear staining, and visualized by fluorescence microscopy. Cell viability was determined in parallel to confirm if cell death was occurring (Figure 13).

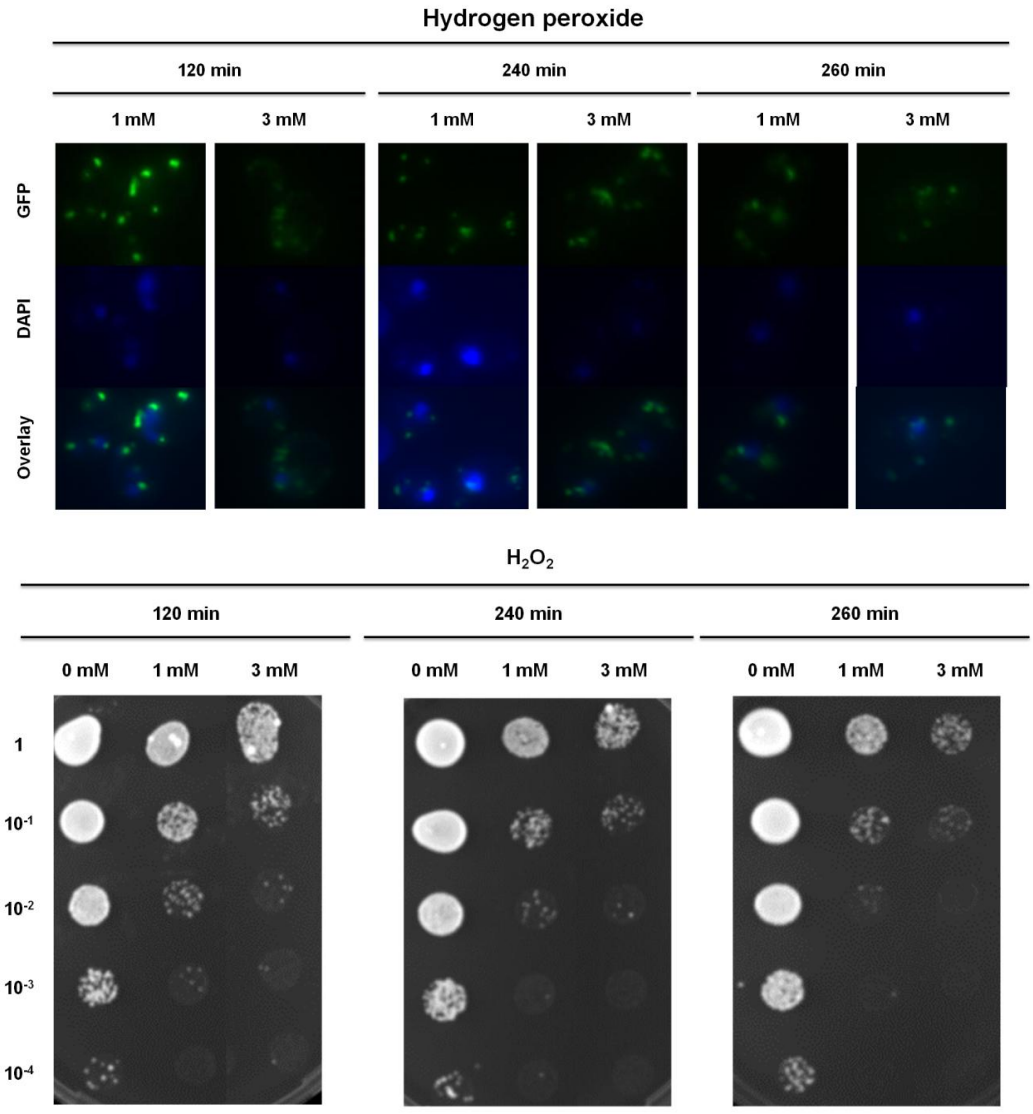


Figure 13 – Aif1p-GFP localization in W303 pAIF1WT-GFP cells after treatment with 1 mM and 3 mM of H_2O_2 during 120 (T120), 240 (T240), and 360 min (T360) and the correspondent dropout tests performed in YPD plates incubated for 2 days at 30 °C to evaluate cell death.

Exposure to H_2O_2 resulted in dose-dependent cell death. However, even after 360 minutes of incubation with different concentrations of H_2O_2, Aif1p-GFP still did not re-localize to the nucleus. It is likely that Aif1p-GFP was not released from mitochondria under these conditions, and by consequence not translocated to the nucleus. As the mode of H_2O_2-induced cell death depends greatly on its concentration and on growth conditions of the culture, we decided to seek a different stimulus to induce mitochondrial outer membrane permeabilization.

4.4. Heterologous expression of Bax *c*-myc protein

Bax, a protein from the Bcl-2 family, is the best-studied member of this group and has important functions in mitochondrial morphogenesis and cell death (Karbowski *et al.*, 2006). Over-expression of Bax in yeast leads to morphological features similar to mammalian apoptosis namely plasma membrane blebbing, DNA fragmentation and others (Ligr *et al.*, 1998). In yeast, several studies highlighted the role of mitochondria in Bax-induced cell death, though as referred in the introduction there is some controversy concerning the type of cell death induced by Bax protein. However, it is well established that a *c*-myc tagged version of human Bax triggers mitochondrial outer membrane permeabilization and release of cytochrome *c* from the mitochondria to the cytosol. However, is referred before t is not known if it also induces the release of Aif1p. The W303 strain expressing pAIF1WT-GFP was therefore transformed with a plasmid expressing Bax *c*-myc, under the control of a tetracyclin-regulatable promoter. Expression of Bax- *c*-myc was inhibited in negative control cells by addition of 1 µg/ml of doxycyclin, an antibiotic member of the tetracycline antibiotics group that represses the promoter. Cells were therefore grown in the presence or absence of doxycyclin to repress or induce expression of Bax (protocol described in 3.5) (Priault *et al.*, 1999). Nuclei were then stained with DAPI to observe if Aif1p-GFP translocates into the nucleus in response to Bax *c*-myc expression (Figure 14).

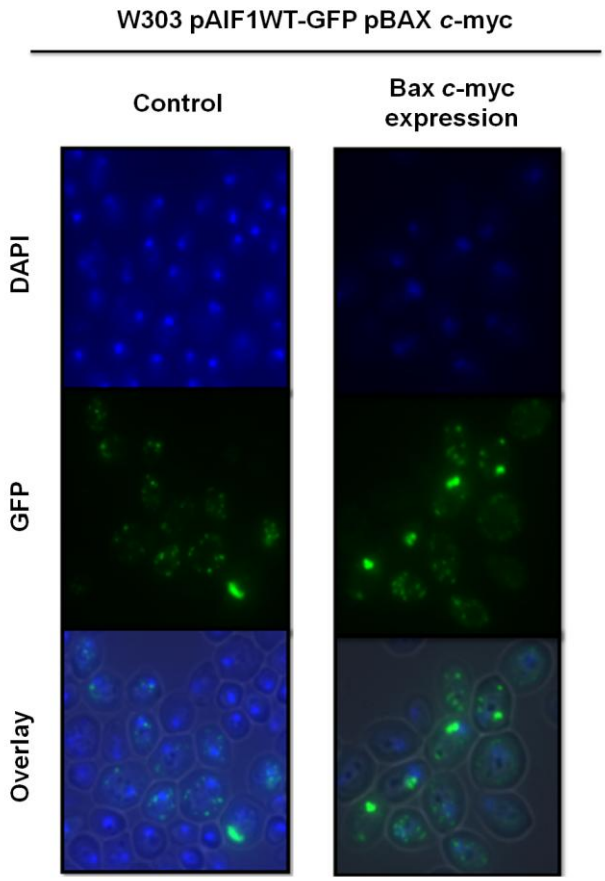


Figure 14 – Aif1p localization in W303 cells expressing pAIF1WT-GFP pBAX *c*-myc. The control corresponds to cells that contain doxycycline in the medium to repress pBax *c*-myc promoter and by consequence repress Bax expression.

Figure 14 shows that, as expected, Aif1p-GFP does not localize to the nucleus in control cells. However, we could also not observe accumulation of Aif1p-GFP in the nucleus even after expressing Bax *c*-myc.

4.5. Aif1p translocation to the nucleus and cytochrome *c* release

The mitochondrion is considered the guardion of several pro-apoptotic proteins, such as Aif1p and cytochrome *c* (cyt *c*). Mitochondria outer membrane permeabilization (MOMP) occurs in the early stages of apoptosis and enables the release of pro-apoptotic proteins to the cytosol (Shimizu *et al*., 2001). MOMP can be triggered by several apoptotic stimuli such as acetic acid, heterologous expression of Bax and others.

We could not observe the release of Aif1p-GFP to the cytosol or its accumulation in the nucleus in the assays described above. Therefore we presumed that either MOMP was not occurring under our experimental conditions, or that the amount of Aif1p-GFP re-localized was much smaller than that in the mitochondria, resulting from Aif1p-GFP overexpression. In the latter case, the strong fluorescence signal from mitochondrial Aif1p-GFP could hinder detection of cytosolic and nuclear protein. We therefore decided to detect MOMP and the re-localization of Aif1p-GFP by performing sub-cellular fractionation by differential centrifugation, to obtain the mitochondrial, cytosolic and nuclear fractions using two apoptotic stimuli, acetic acid and heterologous expression of Bax *c*-myc protein. Aif1p-GFP was then detected by Western Blot using an anti-GFP antibody.

The strain W303 pAIF1WT-GFP was treated with acetic acid as described above in 3.3. Mitochondrial and cytosolic fractions were prepared as described in 3.8.2. The protocol used to obtain the nuclear fraction was the ficoll gradient protocol (protocol l) described above in 3.8.3.1 (Figure 15).

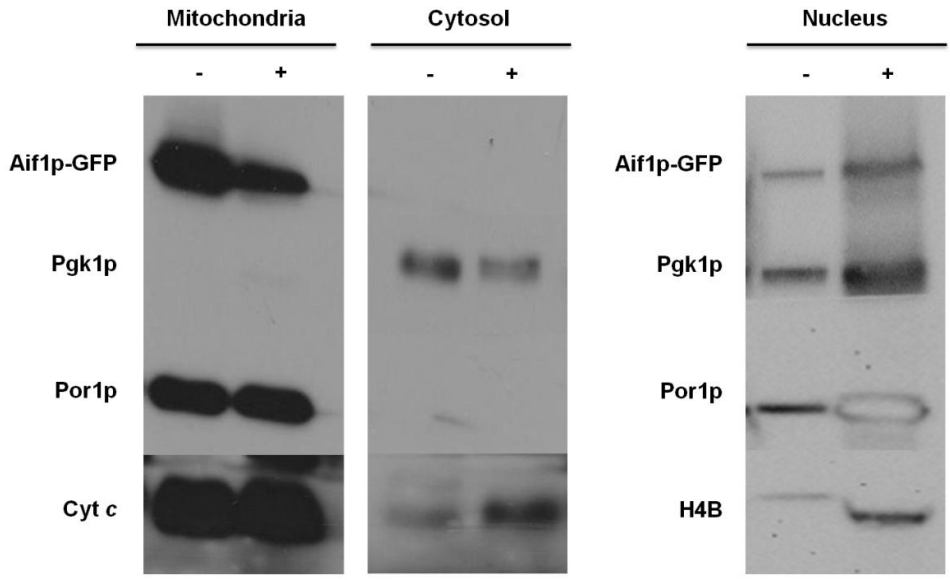


Figure 15 – Western blot of subcellular fractions of *S. cerevisiae* before (-) and after (+) 120 min treatment with 120 mM of acetic acid for the detection of cytochrome *c* and Aif1p cellular localization. The nuclear faction was obtained using protocol I. Loading controls used were cytosolic phosphoglycerate kinase (Pgk1p) for the cytosolic fraction, mitochondrial porin (Por1p) for the mitochondrial fraction, and histone H4 (H4B) for the nuclear fraction.

Although we obtained a fraction enriched in nuclei, as assessed by the enrichment in histone H4B, the protocol used was not efficient. The nuclei fraction

obtained in the 40% ficoll layer did not correspond to pure nuclei, as described by Vongsamphanh *et al.*, 2001. This fraction was contaminated with mitochondria, as we detected a significant amount of Por1p (mitochondrial). Although Aif1p-GFP seemed depleted from the mitochondria, it did not accumulate in the cytosol, and its seeming accumulation in the nucleus is probably due to the higher amount of mitochondria in this fraction (as it had a high amount of Por1p). We could in contrast detect accumulation of cyt *c* in the cytosol of acetic acid-treated cells, indicative of partial MOMP.

We therefore repeated this experiment using a new approach to obtain pure nuclei, the protocol II described in 3.8.3.2 (Figure 16).

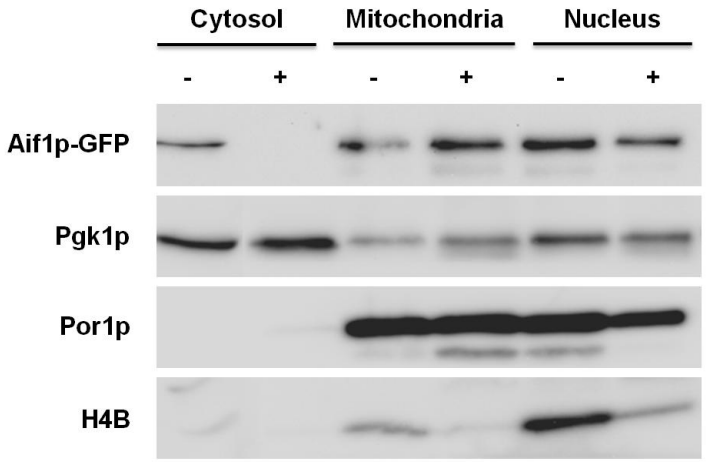


Figure 16 - Western blot of subcellular fractions.of *S. cerevisiae* before (-) and after (+) 120 min treatment with 120 mM of acetic acid for the detection of Aif1p cellular localization. The nuclear faction was obtained using protocol II. Loading controls used were cytosolic phosphoglycerate kinase (Pgk1p) for the cytosolic fraction, mitochondrial porin (Por1p) for the mitochondrial fraction, and histone H4 (H4B) for the nuclear fraction.

The results obtained with this new approach were basically the same as in the first attempt. We also detected Por1p in the nuclear fractions, which indicates contamination with mitochondria, and detected H4B not only in the nuclear fraction suggesting that probably the mitochondrial fractions were also contaminated with nuclei. We also did not observe Aif1p release to the cytosol or translocation to the nucleus. We therefore attempted to optimize the yeast nuclei isolation protocol using

differential centrifugations (protocol III described in 3.8.3.3) (Figure 17). We observed that the majority of nuclei sedimented at 1000 rpm, and most mitochondria sedimented at 3000 and 6000 rpm. However, there was a high amount of Por1p in the 1000 rpm fraction, and thus this fraction was still highly contaminated with mitochondria. We could not therefore satisfactorily separate these two organelles.

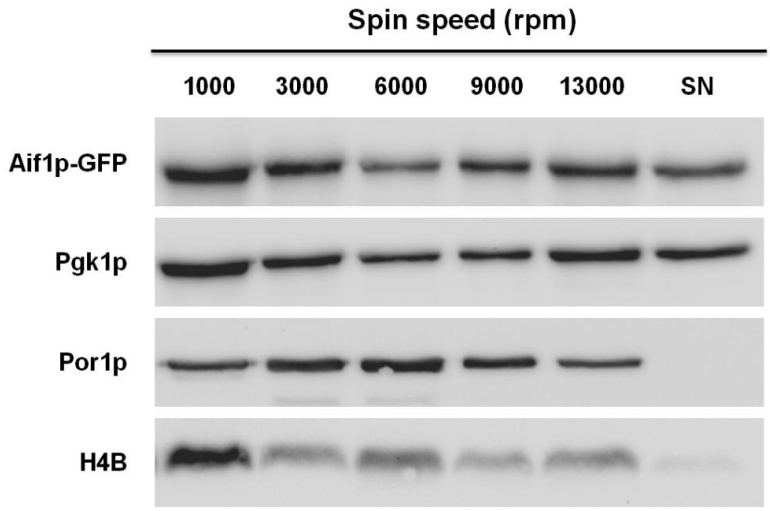


Figure 17 - Western blot of different samples resulted from differential centrifugations (Protocol III). Cytosolic phosphoglycerate kinase (Pgk1p) level was used as control for cytosol for mitochondria the control used was the mitochondrial porin (Por1p) and for the nuclei the histone H4 was used. SN represents the Supernatant.

Since we weren't able to observe Aif1p-GFP release from mitochondria using acetic acid as a MOMP inducer, our next strategy was to perform sub-cellular fractionation in cells expressing Bax protein and evaluate the release of cyt *c* and Aif1p. For this purpose, W303 pAIF1WT-GFP pBAX *c*-myc cells were grown until late exponential phase. Sub-cellular fractionation was performed to obtain purified mitochondria and cytosol (Figure 18).

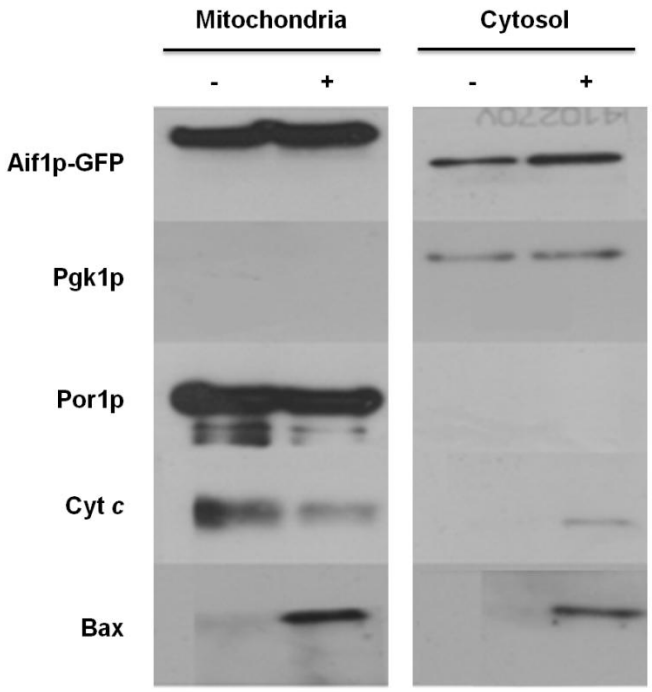


Figure 18 - Western blot of subcellular fractions of *S. cerevisiae* without (-) and with (+) heterologous expression of Bax *c*-myc protein for 14 h, for the detection of cytochrome *c* and Aif1p cellular localization. Cytosolic phosphoglycerate kinase (Pgk1p) levels were used as the loading control for the cytosolic fraction; mitochondrial porin (Por1p) levels were used as the loading control for the mitochondrial fraction. To confirm heterologous expression of Bax protein, Bax detection was performed with an anti-Bax antibody.

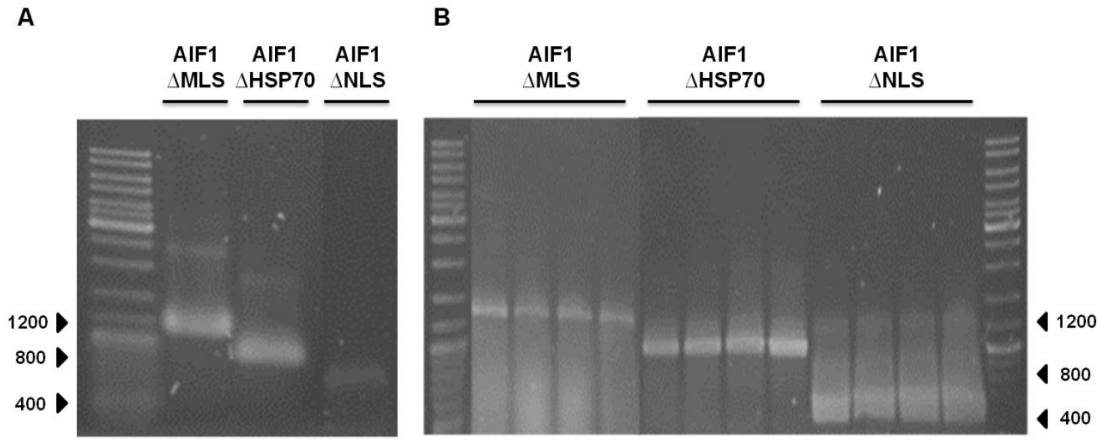
Heterologous expression of Bax protein in yeast leads to cyt *c* release from the mitochondria to the cytosol (Manon *et al.*, 1997). We therefore sought to determine if Aif1p was also released from mitochondria when cells are expressing the pro-apoptotic protein Bax. Western blot analysis showed that heterologous expression of Bax protein induced cyt *c* release in yeast under our experimental conditions, as already described. However, we detected only a minor increase in cytosolic Aif1p-GFP, along with cytochrome *c* release, and only a minor decrease in mitochondrial Aif1p-GFP. These results indicate Bax *c*-myc did induce MOMP and cytochrome *c* release, and likely a small amount of Aifp-GFP release, which is consistent with the results we obtained by fluorescence microscopy. The small differences observed would nonetheless not be sufficient to use this method to uncover proteins necessary for Aif1p to accumulate in the nucleus.

4.6. Map the NLS of Aif1p

The majority of proteins contain localization signals responsible for directing the proteins to a specific location, for example to mitochondria (MLS) or to nuclei (NLS). Since we could not observe Aif1p translocation to the cytosol and then to the nucleus, we decided to pursue another strategy to uncover the soluble factor(s) responsible for transporting Aif1p to the nucleus. For this purpose, we aimed to identify the Aif1p NLS, as expressing an Aif1p domain targeted to the nucleus would greatly facilitate this study.

4.6.1. Construction of *AIF1* fragments and localization of Aif1p

Three different fragments of *AIF1* were amplified by PCR and then inserted into the pYX242GFP plasmid by gap repair. Fragment 1 (AIF1 ΔMLS) starts at nucleotide 78, thus lacking the sequence encoding the MLS. Fragment 2 (AIF1 ΔHSP70) starts at nucleotide 336, lacking the MLS and the sequence homologous to the Hsp70p binding site of human AIF, but containing the sequence homologous to the presumed NLS of human AIF. Fragment 3 (AIF1 ΔNLS) starts at nucleotide 723, resulting in elimination of all the aforementioned domains (Figure 19A). Correct insertion of the fragments into the plasmid was confirmed by colony PCR (Figure 19B) and the localization of Aif1p-GFP in the cells determined by fluorescence microscopy (Figure 19C).



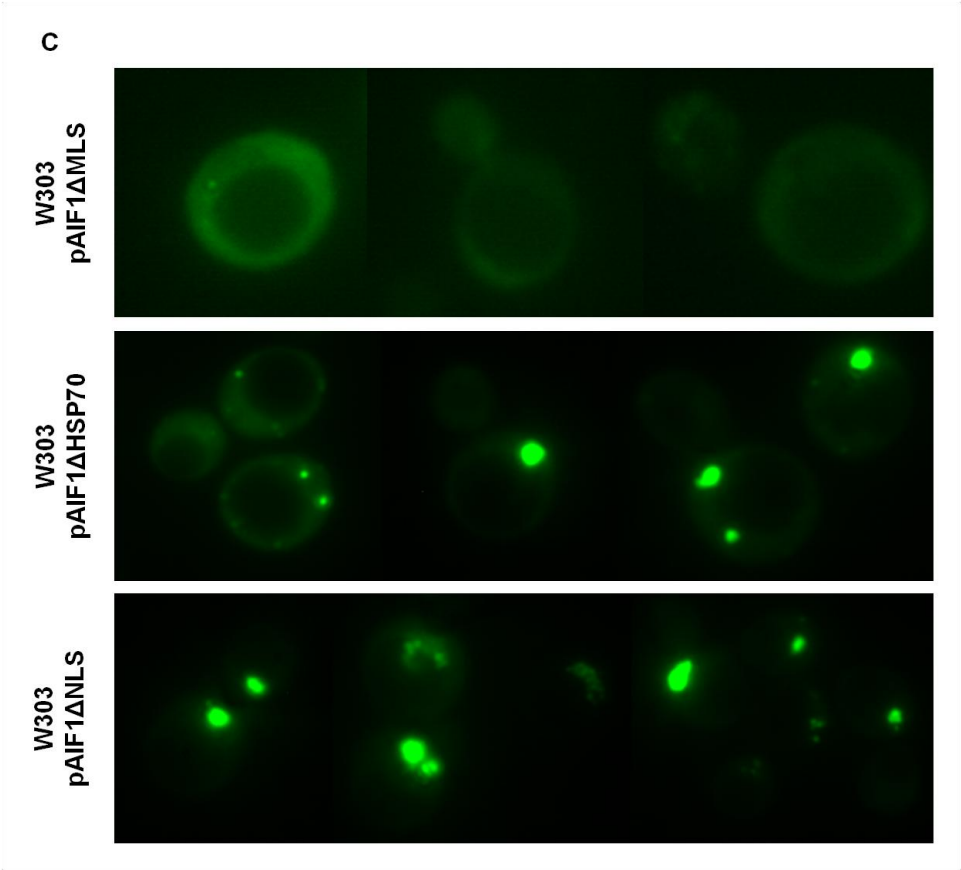


Figure 19 – Confirmation gel of *AIF1* fragments obtained by PCR using as template the pAIF1 from Euroscarf and oligonucleotides 2, 3, 4 and 5 (see Table II) **(A)** Confirmation of the correct insertion of *AIF1* fragments in the pYX242GFP plasmid performed in four candidates by colony PCR using oligonucleotides 5 and 6 **(B)**; Aif1p-GFP localization in W303 cells expressing pAIF1ΔMLS-GFP, pAIF1ΔHSP70-GFP and pAIF1ΔNLS-GFP by fluorescence microscopy **(C).**

As expected, and in contrast to Aif1p-GFP, Aif1p ΔMLS-GFP seems to have a cytosolic localization, showing that deletion of the MLS prevents translocation of Aif1p to the mitochondrion after synthesis in the cytosol. On the other hand, Aif1p isoforms without the MLS and the Hsp70p binding site, and without all three domains (MLS, Hsp70p binding site and the NLS), showed a punctate localization pattern reminiscent of aggregates, which did not co-localize with the nuclei (Figure 20).

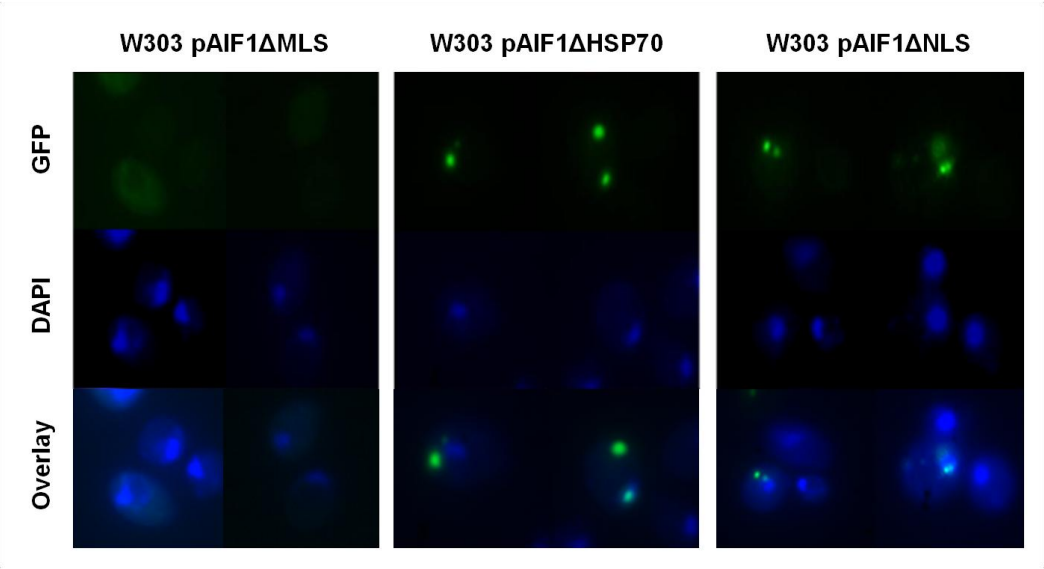


Figure 20 – Co-localization of the three Aif1p fragments with the nucleus. Representative images of W303 pAIF1ΔMLS-GFP, W303 pAIF1ΔHSP70-GFP, W303 pAIF1ΔNLS-GFP cells by fluorescence microscopy, respectively. Cells were incubated with DAPI for nucleus staining.

Hsp70p interacts with AIF, and may stabilize it in the cytosol (Gurbaxini *et al.*, 2003). Deletion of the yeast Aif1p sequence homologous to the Hsp70p binding domain of AIF1 resulted in an aberrant localization and possibly in a misfolded protein. If this was due to deletion of a true Hsp70p binding domain in Aif1p, absence of yeast Hsp70p (Ssa1p) would result in the same phenotype. We therefore transformed an *ssa1*Δ strain and corresponding wild-type with the pAIF1ΔMLS-GFP (Figure 21).

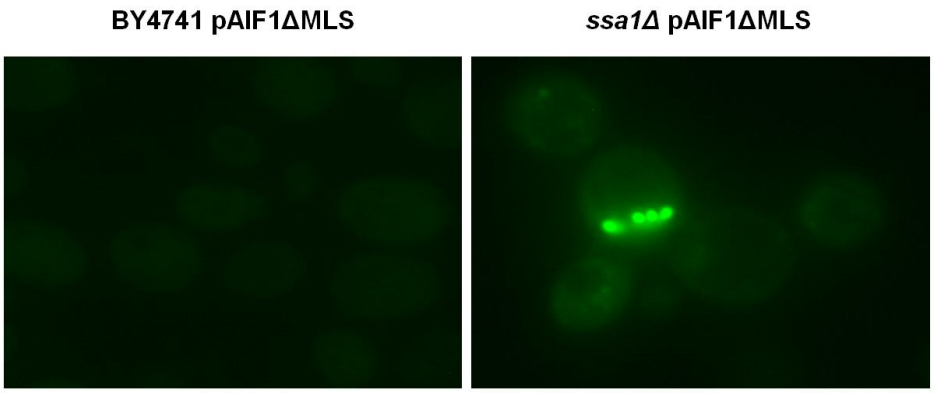


Figure 21 – Observation of aggregates formation in *ssa1*Δ cells expressing pAIF1ΔMLS-GFP in contrast with control cells (BY4741 strain) expressing the same plasmid.

As shown in Figure 21, Aif1p ΔMLS-GFP has a diffuse localization. In contrast, in *ssa1*Δ cells, Aif1p ΔMLS-GFP has localization reminiscent of that of Aifp ΔHSP70-GFP. These results indicate yeast Aif1p may indeed also contain an Hsp70p binding domain, and likely interact with the yeast Hsp70p.

4.7. Protein-protein interactions of Aif1p

We next sough to determine whether Aif1p interacts with Hsp70p in the cytosol, as well as uncover which proteins it may interact with that regulate its localization and possibly its function. Extracts from wild-type and *ssa1*Δ cells expressing pAIF1ΔMLS-GFP were incubated overnight with three different concentrations of antibody against GFP (1, 2 and 5 µg) to immunoprecipitate proteins interacting with Aif1p-GFP (protocol described in 3.9). Extracts from cells expressing pAIF1ΔNLS-GFP were used as a negative control for Hsp70p binding (Figure 23).

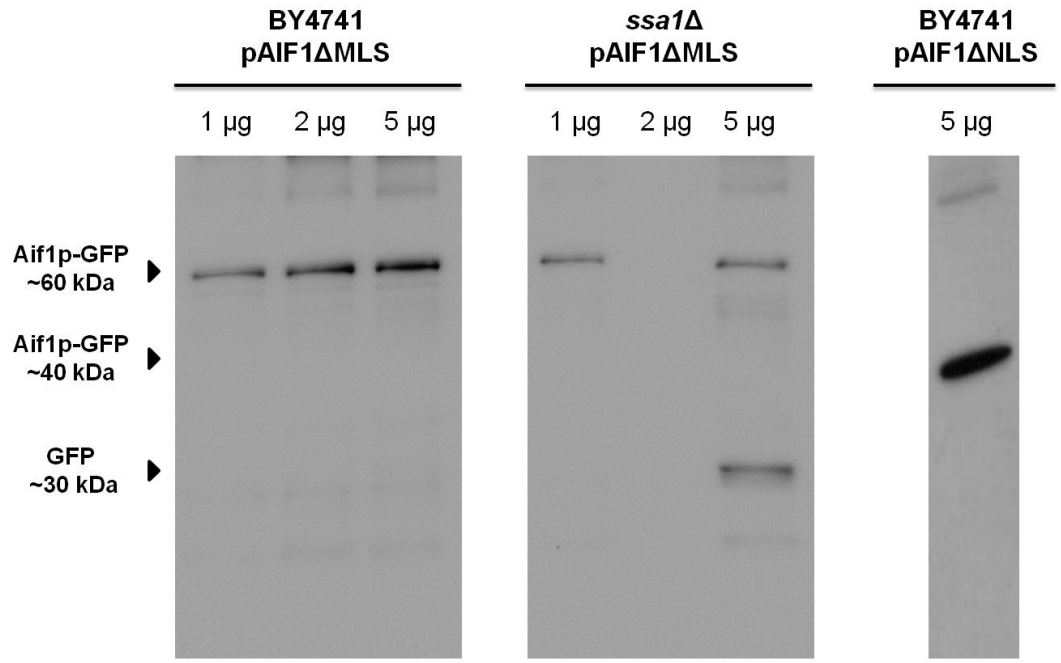


Figure 22 – Immunodetection of Aif1p-GFP obtained by immunoprecipitation of the cytosolic fraction of BY4741 cells expressing pAIF1ΔMLS-GFP or pAIF1ΔNLS-GFP plasmids and *ssa1*Δ cells expressing pAIF1ΔMLS-GFP using a GFP antibody.

As seen in figure 22, all GFP fusions had the expected size, and 5 µg of anti-GFP antibody immunoprecipitated the highest amount of GFP fusions. It was also

apparent that there was less Aif1p ΔMLS-GFP immunoprecipitated from the cytosol of *ssa1*Δ cells, as well as another band with approximately 30 kDa. This presumably corresponds to free GFP, resulting from partial degradation of Aif1p, again suggesting Aif1p requires Hsp70p binding for stability. We next determined the pattern of proteins interacting with Aif1p fusions in the cytosol by silver staining (Figure 23).

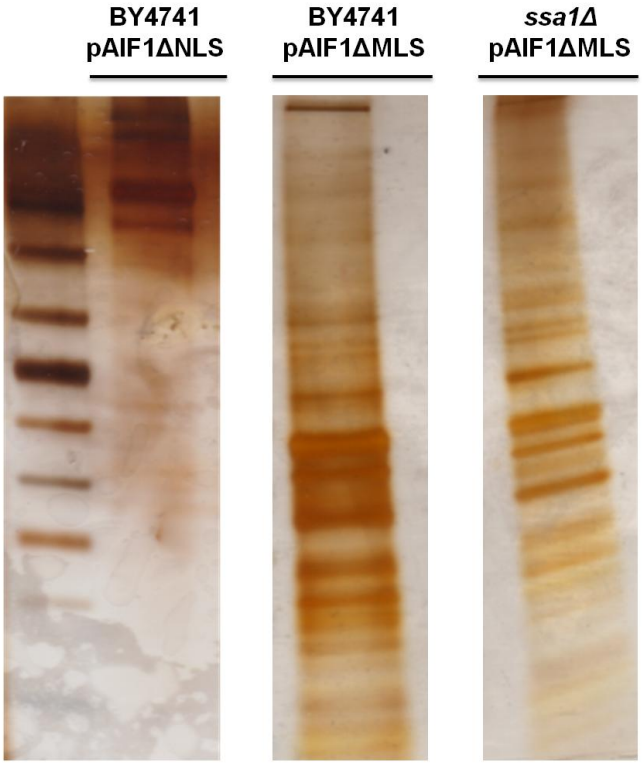


Figure 23 – Profile of Aif1p-interacting proteins obtained by immunoprecipitation and detected by silver staining, in a gradient polyacrilamide gel (4-20%). Cytosol from BY4741 pAIF1ΔNLS-GFP, BY4741 pAIF1ΔMLS-GFP and *ssa1*Δ pAIF1ΔMLS-GFP**.**

We could observe a large number of proteins interacting with Aif1p ΔMLS-GFP in both wild type and *ssa1*Δ cells, which showed a similar profile.

Western blot analysis with an anti-Ssa1p antibody would be required to determine whether Aif1p and Ssa1p interact *in vivo*.

There were much fewer proteins interacting with Aif1p ΔNLS-GFP, indicating the proteins immunoprecipitated with Aif1p ΔMLS-GFP are likely real interacting partners. Mass spectrometry analysis will be required to identify these proteins.

5. DISCUSSION AND FUTURE PERSPECTIVES

Since the discovery that yeasts can commit “cell suicide”, *S. cerevisiae* has been extensively used to study apoptotic cell death, with the objective of gaining insight into poorly understood mechanistic aspects of mammalian apoptosis. In both mammalian and yeast apoptosis, mitochondria have gained great importance due to their ability of propagate the death signals generated within the cell, such as oxidative stress, DNA damage and others (Wang, 2001). Many stress stimuli can trigger MOMP and the release of pro-apoptotic proteins from the mitochondria to the cytosol, such as cytochrome *c* and Aif1p.

Cytochrome *c* exerts its lethal function in the cytosol, which has been well characterized in higher eukaryotes, but mammalian AIF needs to be translocated into the nucleus in order to exert its pro-apoptotic function. The mechanism underlying this translocation is still poorly understood, though it involves a regulated interaction with Hsp70p in the cytosol (Ye *et al.*, 2002). The yeast orthologue of mammalian AIF also plays a role in cell death, and pure Aif1p can degrade yeast nuclei and plasmid DNA. Aif1p, as mammalian AIF, has a mitochondrial localization in normal cells and is translocated to the nucleus after an apoptotic stimulus. However, to our knowledge the mechanism mediating translocation of Aif1p into the nucleus has not yet been studied. For the majority of macromolecules, transport into the nucleus through nuclear pore complexes is an energy-dependent process and normally mediated by the major class of transport receptors, the karyopherins or Kaps. We therefore aimed to elucidate the mechanism regulating yeast Aif1p import into the nucleus, and determine which Kap is responsible for the nuclear accumulation of Aif1p. However, we were unable to establish a robust system to detect accumulation of Aif1p-GFP in the nucleus, and thus we were not able to screen the collection of yeast *kap*Δ mutant strains to determine which were defective in this process.

Several stimuli are described as capable of triggering apoptosis in yeast leading to MOMP and the release of pro-apoptotic proteins such as cyt *c* and Aif1p (Ludovico *et al*., 2002, Vachova and Palkova, 2005, Khoury *et al.*, 2008, Wissing *et al.*, 2004 and Priault *et al.*, 1999). Wissing and colleagues were able to observe the translocation of Aif1p from the mitochondria to the nucleus by fluorescence microscopy, using a plasmid containing the *AIF1* gene under the control of a promoter repressed by methionine. Unfortunately, expressing Aif1p-GFP from this plasmid requires removing methionine from the medium, and minimum levels of fluorescence were observed using the microscope (D. Trindade, personal communication). For this reason, we cloned Aif1p-GFP in pYX242GFP, a multi-copy plasmid, under the control of the strong TPI promoter (pAIF1WT-GFP). This allows the constitutive expression of Aif1p-GFP at high

levels. Indeed, we could observe that cells expressing this plasmid had a strong GFP fluorescence with mitochondrial morphology.

Localization of Aif1p-GFP was studied in cells expressing pAIF1WT-GFP after exposure to acetic acid or H_2O_2, or in cells undergoing chronological ageing or expressing Bax *c*-myc using fluorescence microscopy and sub-cellular fractionation/western blot. Chronological ageing and acetic acid treatment resulted in a total loss of Aif1p green fluorescence levels, presumably because both lead to an increase of intracellular acidification affecting fluorescence emission (not shown). For that reason, the fluorescence microscopy assays were only performed in cells exposed to H_2O_2 and expressing Bax *c*-myc and the cellular fractioning assays in cells exposed to acetic acid and expressing Bax *c*-myc protein. By fluorescence microscopy, we were not able to observe the release of Aif1p-GFP from the mitochondria or its translocation to the nucleus either in response to H_2O_2 exposure or to heterologous expression of Bax *c*-myc. These results suggested that MOMP was not occurring under our experimental conditions, or that the amount of Aif1p-GFP re-localized was much smaller than that remaining in the mitochondria. The signal from mitochondrial Aif1p-GFP resulting from its high overexpression could therefore hinder detection of cytosolic and nuclear protein. To overcome this problem, we are in the process of tagging endogenous Aif1p with GFP in the genome by homologous recombination. Disrupting *AIF1* using short flanking sequences has proven difficult (not shown), probably because this gene resides in a poorly accessible chromatin region, near the telomere. We have therefore amplified *AIF1* tagged with GFP flanked by homology sequences for pAG61 (an integrative plasmid), and cloned it into this plasmid by Gap Repair. We are also in the process of cloning the 3' UTR region of AIF1 downstream of the *URA3* marker in the same plasmid. After digestion with appropriated enzymes, this plasmid will work as a cassette with 1kb of homology up- and downstream of the *AIF1* gene, to allow for more effective homologous recombination in the genome of any yeast strain. This strategy will allow the observation of endogenous Aif1p levels in the different organelles by fluorescence microscopy, without Aif1p overexpression.

As mentioned above, it may have been that we could not observe the release of Aif1p-GFP by fluorescence microscopy because MOMP was not occurring under our experimental conditions, or because the amount of Aif1p-GFP localizing to the nucleus was bellow our detection limits. For that reason, we tried to detect MOMP and the localization of Aif1p-GFP by sub-cellular fractionation in purified mitochondrial, cytosolic, and nuclear fractions. The results of the cellular fractioning and western blot demonstrated that there was some release of cyt *c* both in cells treated with acetic acid and in cells expressing Bax *c*-myc. On the other hand, Aif1p-GFP release into the

cytosol was not observed, suggesting that MOMP is occurring allowing the release of cyt *c* but not of Aif1p-GFP, or that the levels of MOMP achieved were very low, and only the earlier release of cyt *c* was observed. The accumulation of Aif1p-GFP in the nucleus was evaluated by detecting Aif1-GFP in the nuclear fraction. Yeast nuclei were purified using three different methods; however, it was not possible to obtain purified nuclei by differential centrifugation, because it was always contaminated with mitochondria. Consequently, the levels of Aif1p-GFP detected in the nucleus are probably due to the mitochondrial contaminations. It has now been reported that Aif1p is translocated to the nucleus after exposure to hydrogen peroxide in only 25% of cells (Gruhlke *et al.*, 2012). Therefore, we expect that detecting an enrichment of 25% at best of Aif1p-GFP in the nuclear fraction after treatment would not be reliable, and using this method to confirm accumulation of Aif1p-GFP in the nucleus would only be possible in case there were no traces of mitochondrial contamination in the nuclear fraction. Since this would be very technically challenging, we abandoned this methodology.

The major goal of this work consisted in determining which Kap is responsible for the transport of Aif1p to the nucleus. Our intention was to transform mutants in each of the yeast soluble transport receptors (Kaps) with pAIF1WT-GFP and assess the localization of Aif1p-GFP by fluorescence microscopy after apoptosis induction. The mutant with less accumulation in the nucleus would lack the Kap responsible for Aif1p import. However, since it is described that with an apoptotic stimulus, at best 25% of cells show Aif1p translocation to the nucleus, and due to the functional redundancy of this family, there would be the risk of not being able to observe a significant decrease in Aif1p-GFP accumulation in the nucleus in individual Kaps mutants. Mapping the NLS of Aif1p would thus overcome the need for the use of apoptotic stimuli to observe Aif1p translocation to the nucleus, and greatly facilitate this study.

The human AIF protein possesses five typical domains: an N-terminal Mitochondrial Localization Signal (MLS) domain; a FAD and NADH binding domain responsible for its oxireductase activity; an Hsp70p binding domain; a C-terminal end domain; and the Nuclear Localization Signal (NLS) that is located between the aminoacids 367 and 459 (Modjtahedi *et al.*, 2006, Daugas *et al.*, 2000 and Gurbuxani *et al.*, 2003) (Figure 2). Taking this information into account and the homology of hAIF with yeast Aif1p, we cloned different AIF1 isoforms in frame with GFP where different domains were deleted, in order to determine the location of the Aif1p NLS (AIF1 ΔMLS, AIF1 ΔHSP70 and AIF1 ΔNLS). Cells transformed with the different plasmids were visualized by fluorescence microscopy in order to evaluate the localization of the different Aif1p isoforms. As expected, deletion of the MLS domain led to a change of

Aif1p localization from the mitochondria to the cytosol. On the other hand, deletion of the Hsp70p binding domain (AIF1 ΔHSP70 and AIF1 ΔNLS isoforms) led to aberrant localization of GFP, possibly in aggregates. Since deletion of the MLS domain did not result in the nuclear import of Aif1p-GFP, our results suggest that Aif1p translocation to the nucleus is regulated at a step subsequent to Aif1p release from the mitochondria. Our results also suggested that interaction with Hsp70p in the cytosol seems to be important for the correct folding of Aif1p, though additional experiments are required.

Since none of the Aif1p fragments had a nuclear localization, we could not map the NLS of Aif1p. It would be interesting to construct several deletions from the C-terminal end of the gene to map the NLS. Using this strategy, deletion of the Hsp70p binding domain would not occur and Aif1p would not be destabilized. It would still be necessary to delete the MLS domain to guarantee that Aif1p would have a cytosolic localization, and possible an apoptotic stimulus. The location of the hAIF NLS is known, and directed our choice of domains to be deleted. Using further analysis of the homology between the two aminoacids sequences, it would be interesting to delete the corresponding aminoacids in Aif1p and assess if the nuclear accumulation of Aif1p is abolished (once we establish a solid system), in order to discover if the NLS domain is conserved between these organisms.

We cannot discard that AIF does not have a true NLS domain and that the transport of this protein into the nucleus is not dependent on this domain for direct interaction with a transport receptor, but instead is involved in a regulated mechanism involving other proteins, for example the Hsp70p protein. As mentioned above, Hsp70p protein binds to cytosolic hAIF protein and possibly to Aif1p in yeast (see below), acting as chaperone that helps in the folding of the protein and in it stabilization. The NLS domain of a protein is a necessary and sufficient domain to promote translocation of the protein to the nucleus. Since deletion of the MLS did not lead to nuclear import of Aif1p, we hypothesize that either Aif1p has an NLS that is masked and/or regulated by additional proteins, or that Aif1p does not have this domain. It is therefore likely that AIF1 ΔMLS only translocates to the nucleus after apoptotic stimuli. Aif1p and hAIF proteins possess several similarities in terms of structure, function and mode of action, It would thus be interesting to test whether heterologous expression of hAIF protein in yeast complements the phenotype of an *aif1*Δ mutant and whether hAIF-GFP translocates to the nucleus after an apoptotic stimulus (Candé *et al.*, 2002). It would be also interesting to express other hAIF isoforms (described by Gurbuxani *et al.*, 2003) to determine if they have the same localization and phenotype when expressed in yeast cells. Once the Kap(s) responsible for Aif1p nuclear accumulation is identified, it would

be interesting to determine if the same Kap is responsible for hAIF import to the nucleus.

Hsp70p is described as being capable of regulating apoptosis. This protein is involved mostly in the inhibition of apoptosis by blocking Apaf-1/formation of the apoptosome or by stabilization of AIF protein in the cytosol, inhibiting nuclear accumulation of this protein, and thus leading to inhibition of DNA degradation in the nucleus. This protein has been associated with a cytoprotective role against the apoptogenic effects of AIF targeted to the extramitochondrial compartment, as its down-regulation facilitates the induction of apoptosis, whereas its up-regulation leads to inhibition of apoptosis (Ravagnan *et al.*, 2001). Deletion of Hsp70p binding domain (residues 150 to 228) leads to a gain of function phenotype, *i.e.*, facilitates the nuclear translocation of AIF. As mentioned previously, in the course of trying to map the NLS domain of Aif1p, we observed the formation of green fluorescent aggregate-like structures in Aif1p constructs lacking the sequence homologous to the hAIF Hsp70-binding domain, suggesting that yeast Aif1p interacts with Hsp70p. We also observed that cytosolic Aif1p with an intact Hsp70p binding domain localized to aberrant green fluorescent spots in yeast cells lacking Hsp70p protein. This corroborates the idea that binding of Hsp70p protein to Aif1p is required for stabilization and folding of Aif1p and suggests another role of the yeast Hsp70p in the function of Aif1p. We hypothesize that, in the absence of Hsp70p protein or when its Hsp70p binding domain is deleted, cytosolic Aif1p becomes misfolded or unfolded, probably loosing its normal function.

Several stressors lead to misfolded or unfolded proteins, but cells normally ensure that proteins are folded correctly using a variety of chaperones, foldases or lectins. When proteins cannot be restored, misfolded proteins are targeted for processing or degradation (Chakrabarti *et al.*, 2011). In yeasts, there are two major pathways described for protein degradation; the vacuolar pathway and the proteasome pathway. Since AIF1 ΔHSP70 forms spots, possible aggregates, it is possible that Aif1p ΔHSP70-GFP is misfolded and should therefore be partly degraded by one of the degradation pathways. Degradation by the vacuolar pathway does not seem to be occurring in these cells, because the green fluorescent aggregates do not seem to have a vacuolar localization. However, to confirm the exact location of the aggregates, cells expressing the pAIF1ΔHSP70-GFP and pAIF1ΔNLS-GFP plasmids should be stained with Arg-CMAC, a label for the lumen of the vacuole. On the other hand, aggregates could correspond to Aif1p-GFP inside of autophagosomes formed during autophagy. Autophagy is the mechanism by which cells use parts of themselves to survive starvation and stress, limiting cell death by recycling energy and substrates,

and removing damaged proteins and organelles. This autophagic process begins with the formation of a double membrane, the autophagosome. This structure envelops cytoplasmatic content and then fuses with the lysosome/vacuole, releasing the inner content of the vesicle for degradation, recycling in this way the cell's own damaged intracellular machinery. This way, cells can maintain homeostasis during starvation and stress situations. (Codogno and Meijer, 2005 and Hotchkiss *et al.*, 2009) (Figure 24). Aif1p ΔHSP70-GFP as a misfolded protein could be incorporated into the autophagosome in order to be degraded. To test this hypothesis, a western blot can be performed with samples from cells expressing pAIF1ΔHSP70-GFP and pAIF1ΔNLS-GFP to determine the occurrence of autophagy by detecting markers such as accumulation of Atg8p or Atg2p. However, we could not detect any GFP fluorescence in the vacuole, and GFP is normally resistant to degradation in the vacuole (a fact that forms the basis for several autophagy-detecting assays), and thus this hypothesis is unlikely.

Another scenario for misfolded/unfolded proteins is the formation of specialized structures, the aggresomes. These structures result from a saturation of the levels of misfolded/unfolded proteins in the normal proteolytic machinery, which accumulate as ubiquitinated inclusions near the centrosome (Kopito, 2000). Formation of aggresomes was also described in yeast cells (Saliba *et al.*, 2002 and Wang *et al.*, 2009). The localization pattern of aggresomes is similar to that we observed for Aif1p lacking the putative Hsp70p binding domain. To determine whether we are observing aggresomes in cells expressing pAIF1ΔHSP70-GFP and pAIF1ΔNLS-GFP, it would be interesting to incubate cells with MG132, a proteasome inhibitor that increases the formation of aggresomes (Saliba *et al.*, 2002). Another approach would be performing co-localization studies of the "aggregates" with the spindle pole body (SPB), the prototype of the centrosome in yeast, because normally the aggresomes are located near the centrosome. For this purpose, we could

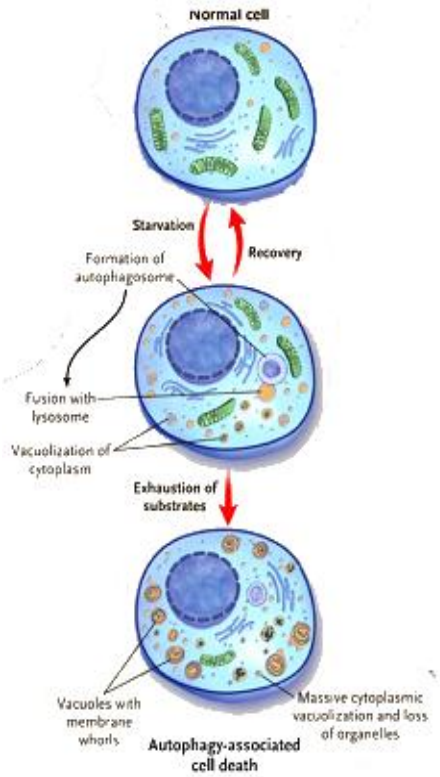


Figure 24 - Representation of the molecular and morphological processes associated with autophagy (Hotchkiss *et al.*, 2009)

use cells expressing BFP-tagged components of the SPB (Wang *et al.*, 2009 and Moens *et al.*, 1971).

To uncover which proteins interact with Aif1p-GFP in the cytosol, we performed an immunoprecipitation of the cytosol from cells expressing the pAIF1ΔMLS and pAIF1ΔNLS (control) (Figure 23). Our results show that the Aif1 ΔMLS protein specifically interacts with multiple proteins in the cytosol (as the most immunoprecipitated proteins do not interact with the Aif1 ΔNLS protein). Taken together, our results thus suggested that Aif1p interacts specifically with several proteins in the cytosol, including with the Hsp70p protein. To confirm the interaction of Aif1p with Hsp70p protein, we will next detect the presence of Hsp70p in the immunoprecipitated fraction with an antibody against Ssa1 (Hsp70p). We will next optimize this protocol in order to identify Aif1p-interacting proteins by mass spectrometry, both in cytosolic extracts from cells without and with an apoptotic stimulus. We will first elute interacting proteins with a salt gradient to distinguish which bands represent proteins with a strong interaction with cytosolic Aif1p, and to determine the salt concentration for which we eliminate most unspecific bands. Bands and/or the elution fractions can then be analyzed by mass spectrometry for protein identification. Identification of proteins that interact with cytosolic Aif1p before and after an apoptotic stimulus should provide valuable insight into the regulation of this protein and its pro-apoptotic effect.

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